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CHAPTER 7

Supercritical fluid chromatography (SFC) – global perspective and applications in lipid technology

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A. Introduction

Supercritical fluid chromatography (SFC) is not a new analytical or processing technique, having its origins as far back as 1962, as reported by Klesper, Corwin, and Turner (1962) in the *Journal of Organic Chemistry*. This initial study, although embodying the basic principles of chromatography as we understand it, was totally different in experimental execution to modern SFC, and used an approach not altogether dissimilar to the classic chromatography studies of such pioneers as Tswett, Day or Zechmeister (Heftmann, 1975). Using fluorocarbon eluents and conventional gas chromatographic stationary phases, these researchers demonstrated a simple separation of metalloporphyrins by removing the column packing material, after displacement of the porphyrin moieties had been accomplished by a glass wool plug placed at the head of the column! Whereas this crude SFC technique is somewhat analogous to flash chromatography, it demonstrates that SFC can be utilized for separating lipids to advantage in a multitude of ways other than by the familiar high resolution techniques.

Indeed, SFC has been employed using frontal, displacement and elution development modes, as characterized by the differential migration experiments of Giddings *et al.* (1969) in the late 1960s. Historically, a breakthrough occurred in the review paper of Novotny and co-workers (1981), in which elements of high performance liquid chromatography (HPLC) injection were blended with open tubular gas chromatographic (GC) columns to facilitate high resolution separations not previously attainable by either HPLC or GC. In the early 1980s, commercial SFC instrumentation became available, initially in the packed column mode, and quickly followed by open tubular column-based SFC instruments and column technology. The

latter mode, generically referred to as capillary SFC, was popularized by a series of international conferences held on the Wasatch front in Utah by Markides and Lee (1988, 1989). These "application" books contained many examples of SFC applied to samples containing lipids.

It should not be surprising that SFC would find a natural niche in lipid analysis. Basic solubility studies of lipids in supercritical carbon dioxide (SC-CO_2), the most commonly used supercritical fluid (SF), indicate that lipids have relatively high solubilities in SC-CO_2 (Stahl *et al.*, 1980). This is supported by the many studies devoted to characterizing the extraction of natural oils and fats using SC-CO_2 (Friedrich and Pryde, 1984; Stahl *et al.*, 1980; Eggers and Stein, 1984). Several excellent review articles since 1995 (Smith and Hawthorne, 1997; King and Snyder, 1997; King, 2002a) and books (King and List, 1996; Caude and Thiebaut, 1999) are available, which describe the application of SFC and supercritical fluid extraction (SFE) to lipids.

This review will describe more recent developments in applying the principle of SFC in a "global" sense, to separate, characterize and process lipid-containing materials. There are sections devoted to high resolution analytical-based SFC separations, sample preparation, preparative or plant scale SFC, and the utility of SFC for physicochemical property determination. The value of SFC is too often measured only in terms of its value to the analytical chemist concerned with high resolution chromatographic separations. This chapter will try to expose the reader to an expanded spectrum of uses for SFC, many of which have not been discussed in past reviews.

The research described in this chapter is also biased towards developments in the field since 1995. There are a number of available reviews (Schoenmakers, 1990; van Oosten *et al.*, 1991; Bartle and Clifford, 1992; Sandra and David, 1996) on the SFC of lipids which describe earlier developments in the field. These include Laasko's summary (1992) in a previous *Advances in Lipid Methodology*, Matsumoto and Taguchi's review (1994) in *Lipid Chromatographic Analysis*, and Lesellier's *et al.* (1993) focus on carotenoid analysis. A reduced emphasis has been placed on the coupled methodology of SFC with SFE, since the commercial availability of instrumentation to utilize this technique is, at best, limited. This fact, when coupled with the high level of sophistication required to use SFE/SFC, has largely relegated its use to academic laboratories, hence routine utilization of this technique has been lacking. Nevertheless, specific applications of the technique will be noted later, and the reader is referred to an excellent tome by Ramsey (1998), which provides an updated review of "on-line" SFE/SFC methodology. The bi-annual reviews in *Analytical Chemistry* authored by Chester, Pinkston, and Raynie (1990–2002) are also a useful source of information on past and present developments in SFC and allied techniques.

Unless otherwise mentioned, it will be assumed in this review that the supercritical fluid utilized is SC-CO_2, perhaps with selected co-solvents in the

case of packed column SFC. Today, neat carbon dioxide is almost extensively used as opposed to helium-imbibed sources, since numerous studies (Raynie and Delaney, 1994; King *et al.*, 1995; Gorner *et al.*, 1990) in both SFC and SFE have indicated that the cylinder composition containing a helium head pad varies over the time of its use. This compositional change has resulted in concomitant changes in chromatographic retention parameters and solute solubility in the SF. Similiar considerations also apply to the case of using co-solvent-laced delivery cylinders, and it is preferable to add a co-solvent dynamically in the case of SFC or SFE.

As will be noted later, the use of a critical fluid (sub- or -super), such as SC-CO_2 in SFC, can have unexpected benefits, even when not used under SF conditions. An excellent perspective on the "universality" of using critical fluids in chromatography is provided by Chester (1997), in which he describes the generous one-phase region available for chromatography using SFs such as SC-CO_2. Therefore, SC-CO_2 can be used to benefit below its critical temperature, T_c, and pressure, P_c, in the subcritical or enhanced fluidity regions (Lee and Olesik, 1994). Besides the thermodynamic-based flexibility afforded by the use of supercritical fluid media, solute-fluid mass transport properties can also be enhanced by imbibing a conventional liquid phase with a quasi-liquid fluid such as CO_2 under pressure. One obvious advantage is to increase the fluidity of the mobile phase (i.e. high pressure CO_2 dissolved in a conventional liquid) to control both solute retention and enhance mass transfer and the speed of analysis. An excellent example is the work on enhanced fluidity liquid chromatography (LC) by Olesik and co-workers (Phillips and Olesik, 2002).

There is a close analogy between developments and results in analytical scale methodology and the use of SFs for processing purposes. Valuable data are gained from both of the above disciplines that can be utilized in and transferred to the other. For example, solubility data for lipid solutes in SFs are crucial in SFC separations, particularly when they are scaled up to a preparative/process level (Nicoud *et al.*, 1999). A later section gives examples of the use of SFC for physicochemical property determination, a field pioneered by Schenider and co-workers (Bartmann and Schneider, 1973; van Wasen *et al.*, 1980; Wilsch *et al.*, 1983) with respect to lipid solutes.

Finally, a key question is why we should use SFs for analysis or processing purposes? Besides the often-cited benefits in terms of chromatographic theory and application (Giddings, 1965) that SFs afford, there is the issue of environmental and laboratory compatibility. The use of SFs, especially CO_2-based methodologies, can reduce substantially dependence on organic solvents in solvent extraction or high performance liquid chromatography (HPLC). This is true not only when substituting SFE for solvent-based extraction methods (King, 2002b), but also for SFC, as has been amply illustrated by its use in the pharmaceutical industry (Berger, 1995). Most SFC applications for lipids do

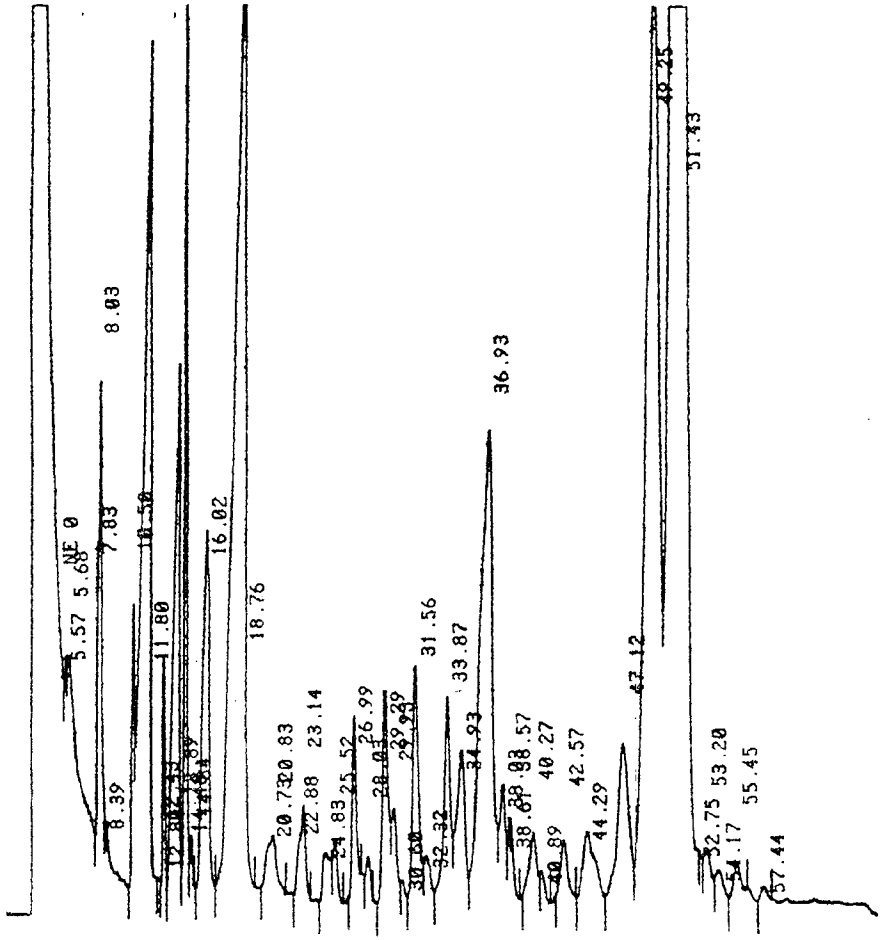

Figure 1. Capillary supercritical fluid chromatography (cSFC) of saw palmetto extract.

not require the collection, disposal, or recycling of the organic solvents utilized in other techniques. Another advantage of SFC-based techniques is that they can provide substantial information with minimal effort. Figure 1 shows a pressure programmed capillary SFC profile of the components in a saw tooth palmetto berry extract used for nutraceutical purposes. This chromatographic result was achieved simply by dissolving an extract in 1–2 mls of solvent, and then directly injecting it onto the SFC column. As one can see, this mixture of fatty acids and sterols is nicely separated in one hour without resorting to any sample preparation.

B. Utility of SFC

This section focuses on the diversity of SFC as both a high and low resolution chromatographic option, its role in both analytical and preparative (production) scale processing of lipids, and the non-analytical use of SFC to determine physicochemical data. Since this is a review, a discussion of the fundamental aspects of SFC is not warranted; there are several texts and publications that fulfil this role. The classic book by Lee and Markides (1990) devoted largely to capillary SFC is recommended, as is Berger's (1995) tome on packed column SFC. There are other lengthy reviews on the basics of SFC (Petersen, 1990; King *et al.*, 1993a). A fairly recent review of SFC instrumentation has also been provided by Erickson (1997).

From an analytical perspective, SFC is frequently defined in terms of column technology; that is, capillary versus packed columns. Considerable debate ensued about the relative merits of both of these approaches during the 1980s and into the early part of the 1990s, but in truth both have their place in SFC for the separation of lipids. Capillary SFC is accomplished using silica-based open tubular columns, 50–100 micron in diameter, at lengths ranging from 5–25 m. Polymeric liquid phases (i.e. functional polysiloxanes that are chemically-bonded at 1–5 micron film thickness) predominate in capillary SFC, while packed column SFC is characterized by the use of 1–10 micron functionalized silica-based packings, where the nascent surface activity has been minimized by the using of special end-capping techniques on the residual hydroxyl groups. Owing to the weak elutropic strength of SC-CO_2 as a mobile phase, it is not uncommon to add a modifier (co-solvent) to the SC-CO_2 to improve peak symmetry. Polymer resin packing material has also been used more sparingly in SFC because of its lower adsorptive properties, but this advantage is offset by its mechanical instability at higher operational pressures (King *et al.*, 1988). It is also not uncommon in packed column SFC to use more than one column in series to increase the overall plate count required for more demanding separation problems.

Figure 2 shows generic schematics of both capillary (A) and packed (B) column SFC. Both techniques can include a co-solvent pump; however, this is more difficult to facilitate in the capillary mode than in packed column SFC, where inclusion of a co-solvent pump is more critical. Heat tracing of these systems is important since the occurrence of multiple phases may be deleterious to the resultant chromatography (Page *et al.*, 1992), but this is difficult to achieve outside the confines of the column oven. The most popular detectors in packed column SFC are the ultraviolet (UV) or evaporative light scattering detectors (ELSD) for lipid solutes, while in capillary SFC the flame ionization detector (FID) reigns supreme (King *et al.*, 1993a). When detecting high molecular weight lipids, the problem of solute solubility in the decompressing SF fluid is circumvented by using either a pressurized detection cell, or a

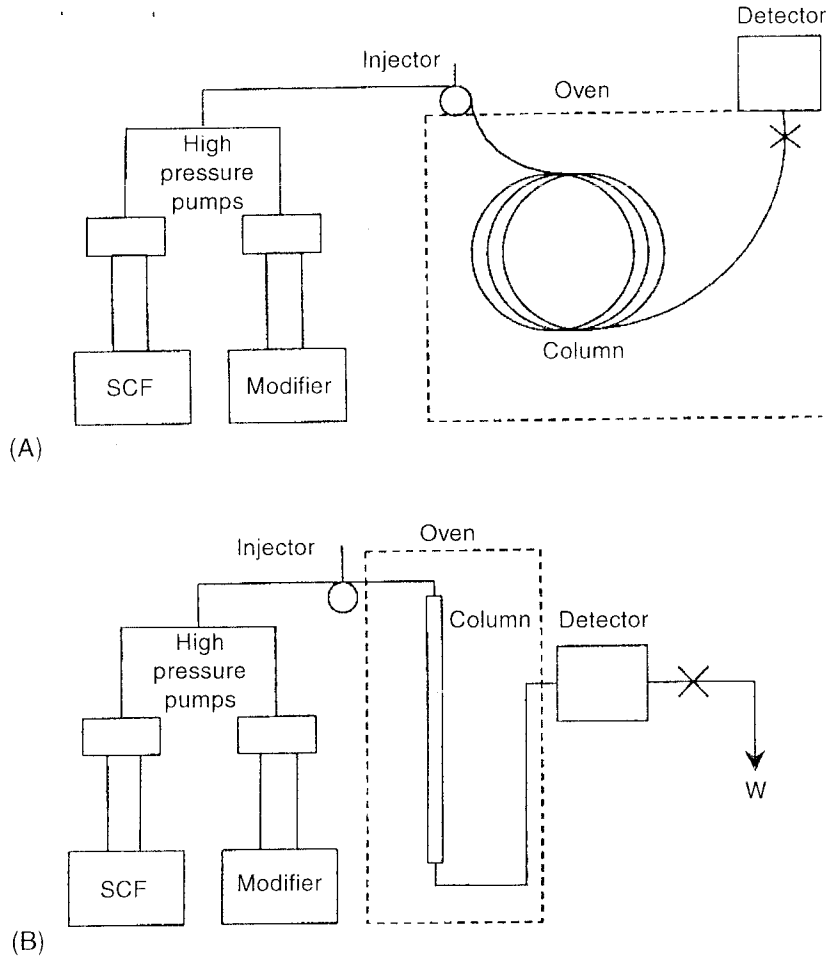

Figure 2. Basic instrumentation components in a (A) cSFC and (B) packed column SFC systems.

capillary "jet" that is heated to assist in the nebulization of the fluid into the detector proper. Such considerations also apply when mating SFC with spectroscopic-based detectors such as mass spectrometry (MS) or Fourier Transform infrared detection (FTIR) (Calvey, 1996).

1. Advantages of SFC relative to GC and HPLC

Analytical SFC is often characterized as a hybrid technique that encompasses some of the features of both GC and HPLC (King *et al.*, 1993), but we shall avoid this approach here by emphasizing the advantages the technique relative

to GC and HPLC. With respect to the use of GC for lipid analysis, SFC can facilitate separation the separation of non-volatile, thermally-labile compounds without resorting to the derivatization required in GC analysis. Indeed, analytical SFC separations have been achieved on solutes up to and above 1000 in molecular weight using either capillary or packed column SFC. Relative to HPLC, SFC affords the use of the flame ionization detector (FID), which is compatible with the non-chromaphoric properties of many lipid solutes, and a substantial reduction in the use of organic solvent. The use of SFC can save the analyst considerable time by reducing not only sample preparation, but also actual analysis time when considering the analytical information that is required. This will be illustrated later in the applications section. Although high chromatographic efficiencies are possibly with SFC, rivalling those possible with GC or HPLC, it is generally acknowledged that GC is the better choice for acute lipid separations, such as critical pairs. Ultimately the power of SFC is linked to the control of separation efficiency and resolution through regulation of the mobile phase pressure and temperature.

Solute retention in SFC is to a first approximation governed by the molecular weight of the solutes being separated. White and Houck (1985) showed that the solute retention time in SFC was an approximate linear function of solute molecular weight for the separation of mixed lipid species. Hayes and Kleiman (1996) extended this approach when separating triglyceride moieties of new crop seed oils. This relative regularity of solute retention sequence can be used to identify species lipid compounds as well as estimate their molecular weights (Hayes and Kleiman, 1996a). Figure 3, taken from the studies of Borch-Jensen

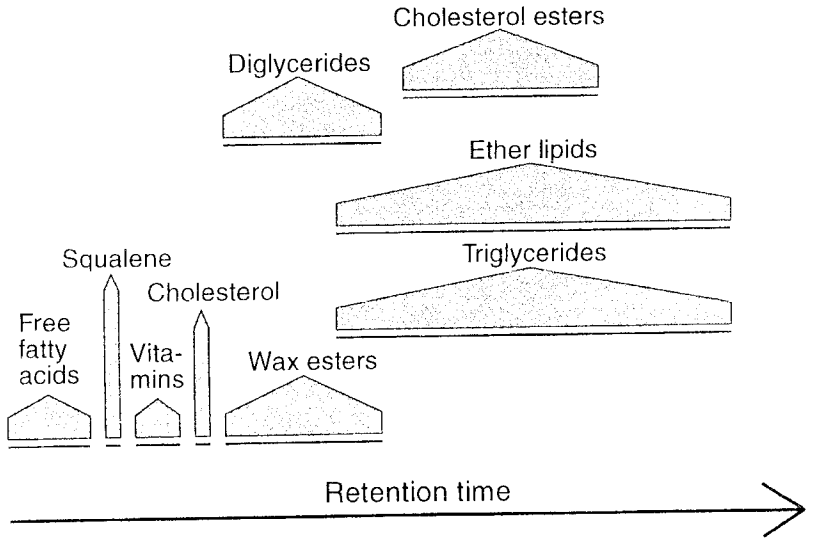

Figure 3. Retention trends for various lipid classes in cSFC.

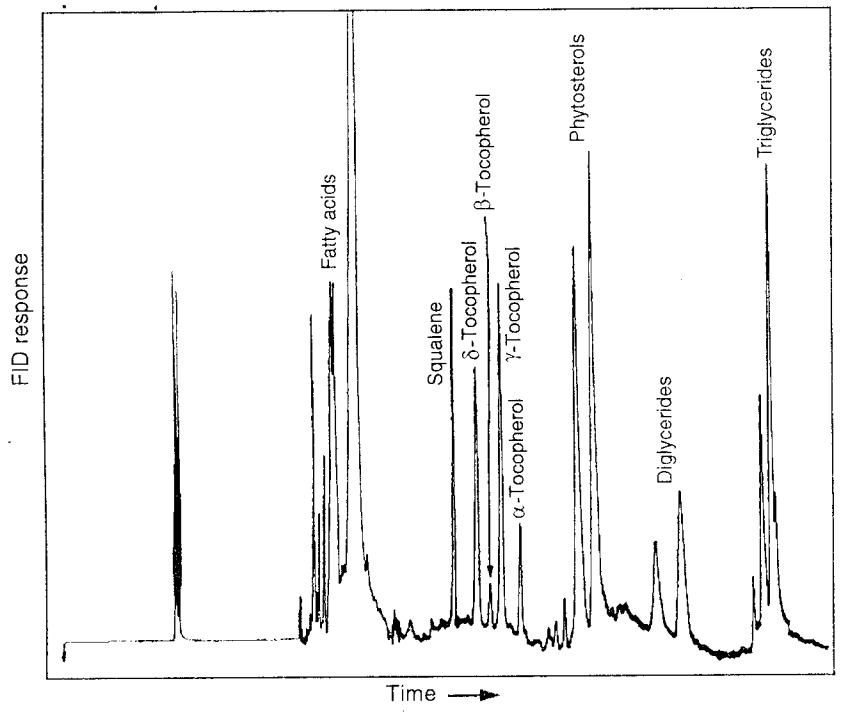

Figure 4. cSFC-FID analysis of the components in a deodorizer distillate.

and Mollerup (1996a), indicates that the specific classes of designated lipids can be separated based on their molecular weights quite conveniently by SFC up to molecular weights of 1200 Daltons. This solute retention scheme is quite convenient for monitoring the conversion of lipids from one species to another; for example, the conversion of triglycerides to species with a lower molecular weight, such as monoglycerides or fatty acid methyl esters (Jackson and King, 1996; Snyder *et al.*, 1996) as depicted in Figure 4. Chromatographic separations embracing this large a molecular weight range with the reported resolution are difficult to achieve by GC or HPLC alone.

The use of SFC alone also has advantages in terms of avoiding tedious sample preparation (Figure 5). Here the determination of cholesterol in a fish oil capsule is possible without any prior sample preparation other than dissolution of the oil in hexane for purposes of injection. The inscribed density program on the horizontal axis affords a nice separation of cholesterol (as well as α-tocopherol) from the background triglycerides that constitute the main components of the fish oil. The separation shown in Figure 5 takes about an hour and 25 minutes in total, which might seem inordinately long. However,

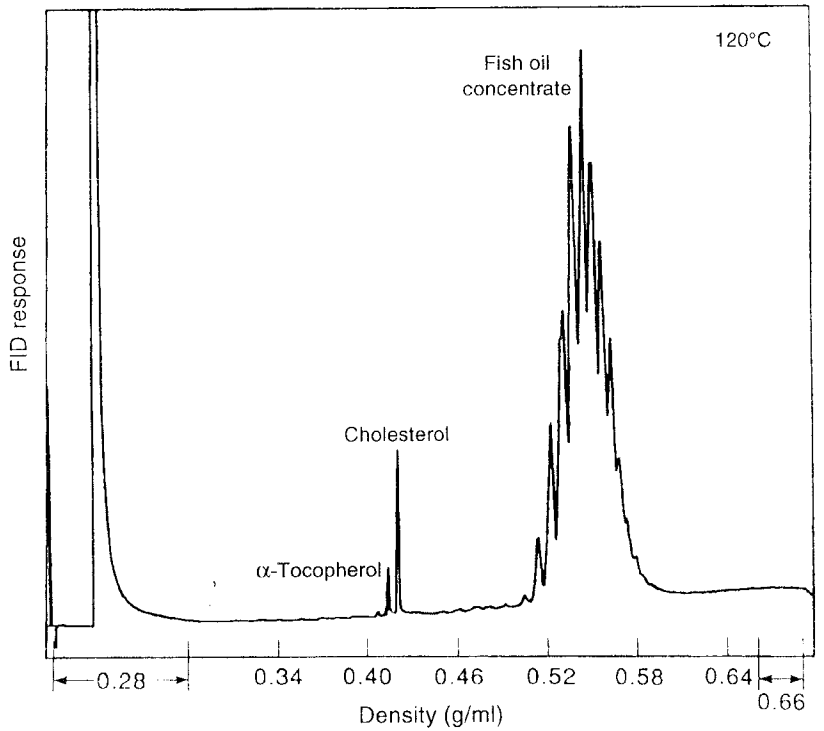

Figure 5. cSFC of lipid components in a fish oil capsule.

when one considers that traditional sample preparation could take even longer, the advantage of the separation in Figure 5 becomes apparent. The large separation factor between cholesterol and the triglycerides further suggests that this separation could be optimized with respect to time and, indeed, a 30 min separation is possible by adjusting the mobile phase density program (King, 1990).

2. *SFC as a coupled technique*

The coupling of SFC with analytical SFE, particularly in the capillary mode, has been demonstrated by numerous investigators. As noted previously, such coupled methodologies are not easily accomplished and require an analyst with skill in both analytical SFE and SFC. Research conducted in the early 1990s resulted in several instrument vendors offering SFE-SFC systems, but reproducibility, mechanical installation difficulties and injection artifacts limited the routine use of this methodology. However, for some specific cases involving

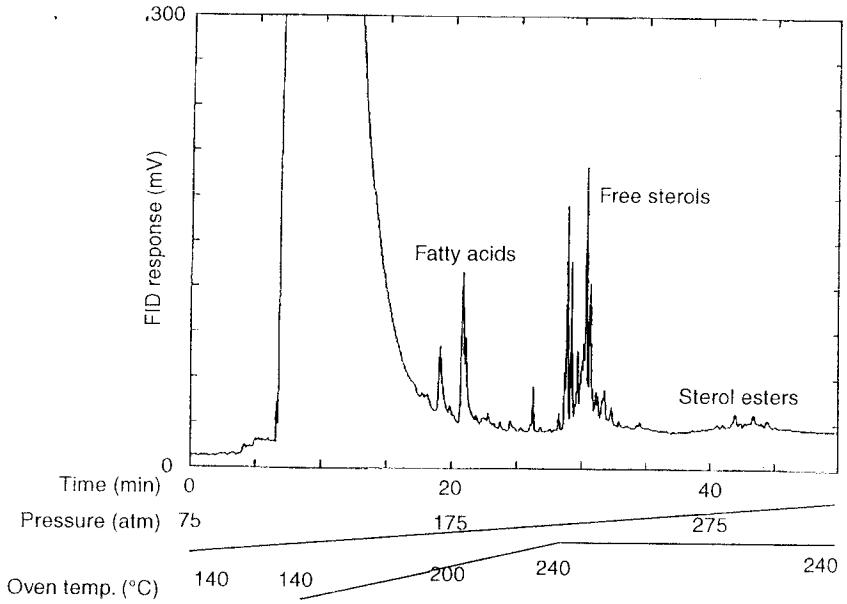

Figure 6. SFC of lipophilic components extracted from a freeze-dried hamster faeces.

matrices containing lipid solutes, coupled SFE-SFC proved a very facile technique, particularly for the characterization of small samples. Figure 6 shows the separation of the free fatty acid, sterols, and sterol esters in a single hamster faeces by capillary SFC using the inscribed pressure-temperature program on the horizontal axis (Pinkston *et al.*, 1991). This extraction and separation sequence was accomplished in under an hour's time and provided valuable metabolic information on the fate of lipids in the hamster's digestive system. Similarly, King (1990) utilized SFE-SFC for the analysis of pheromones and cuticle lipids from beetles, which were extracted using SC-CO_2 at 40°C at 200 atmospheres. As shown in Figure 7, the active pheromone constituent elutes very early in the extended chromatogram, eventually being followed by the cuticle lipid constituents, which are predominantly wax esters. In this case, a single beetle was isolated for extraction by inserting the insect in a vial, and placing the vial briefly into a refrigerator (to lower the insect's metabolic rate) prior to its placement in the extraction cell. Unfortunately, the beetle did not survive the quoted (Figure 7) extraction conditions!

Nam and King (1994) incorporated a microbore SFC column as an intermediate step for cleaning up on-line SFE extracts containing trace levels of pesticides in meat tissue. A 100 × 1 mm C_8 column was connected between the SFE cell and a conventional GC megabore capillary column for separating and detecting the organochlorine or organophosphorus pesticides by hetero-element

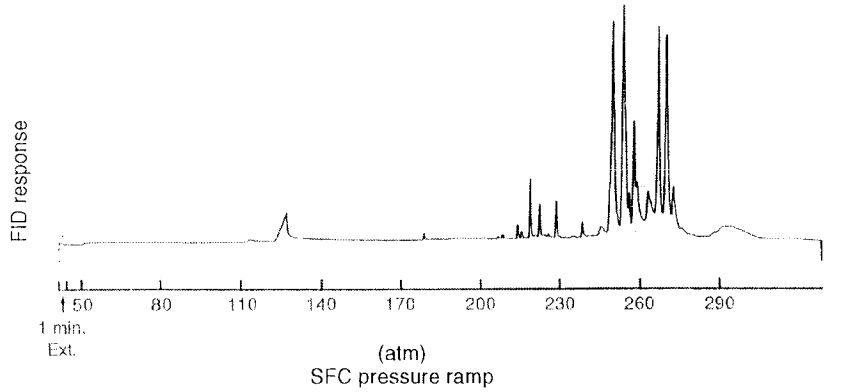

Figure 7. On-line SFE/cSFC of a CO_2 extract from a single fruit beetle. Extraction pressure: 200 atm; extraction temperature: 45°C; extraction time: 1 min; extract cryofocused.

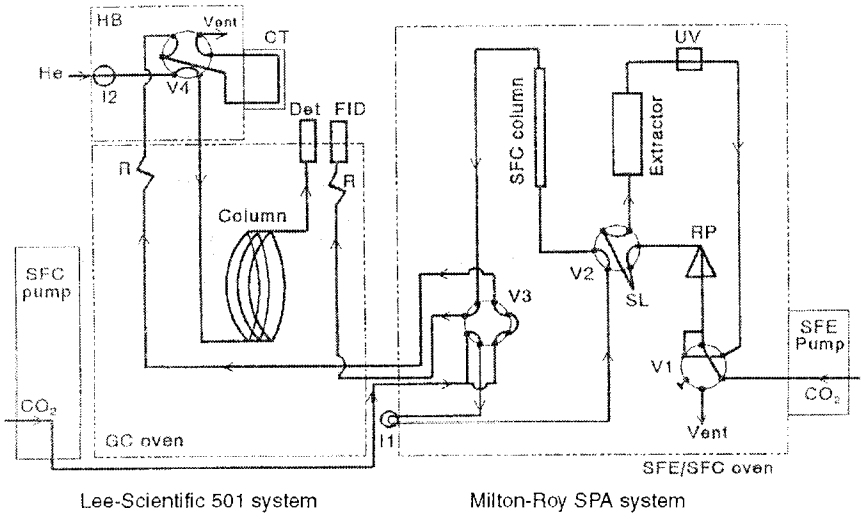

Figure 8. A coupled SFE/SFC/GC system. V(1–4): switching valves; R: flow restrictor; Det: ECD or NPD; RP: recirculating pump; SL: sampling loop; I1: SFC injector; I2: GC injector; CT: cryogenic trap; HB: heated block; He: helium.

GC detectors. A schematic of the experimental system is illustrated in Figure 8. The valves are used in a sequential manner to divert the SC-CO_2 first into the extraction cell, and then into the SFC column, which afforded the separation of the pesticides as a focused analyte band separated from the interfering lipids (fat). Excellent quantitative results at the ppb level were achieved using this coupled SFE-SFC-GC technique.

3. *Supercritical fluids as a mobile and stationary phase modifier*

Supercritical fluids have been predominantly used in their neat form as chromatographic eluents with the possible inclusion of co-solvents as mobile phase modifiers, particularly in the case of packed column SFC. The desirability of maintaining a single coherent phase in this case for the mobile phase has been stressed by Page *et al.* (1992) and others, based upon chromatographic reproducibility and optimization of plate height for maximum peak resolution. However, despite these arguments, mobile phases consisting of two discrete phases (e.g. a co-solvent in SC-CO_2 phase separates) have been used to advantage to accomplish separations.

Other than phase separations, inclusion of SC-CO_2 confers a decrease in viscosity in the mobile phase, which is the basis of SFC's superiority over HPLC in the generation of low theoretical plate heights (King *et al.*, 1993a). The impact of this basic physicochemical property on solute mass transport in the chromatographic system (i.e. solute diffusion coefficients) is apparent when CO_2 is blending with a conventional liquid solvent. This concept has been given the term of "enhanced fluidity" chromatography by Olesik and co-workers (Phillips and Olesik, 2002) and has been shown to offer several advantages.

For example, replacement of a substantial quantity of an organic solvent by CO_2 has both cost and environmental advantages. Secondly, the use of SC-CO_2 in place of a liquid solvent can be used to regulate retention and accomplish separation (Yuan and Olesik, 1997). Thirdly, the weak elutropic strength of

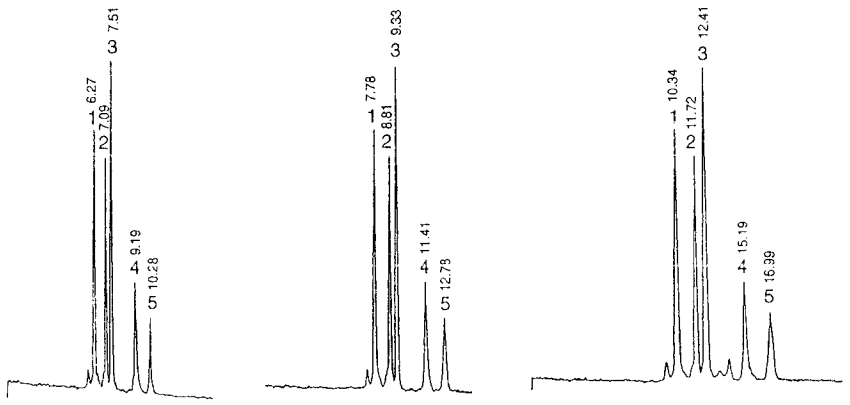

Figure 9. Effect of mobile phase composition (% SC-CO_2 – organic solvent) on retention volume of solutes in calibration mixture. A: 100% methylene chloride; B: 80% methylene chloride, 20% SC-CO_2; C: 60% methylene chloride, 40% SC-CO_2. Conditions: Jordi gel 500 Å (250 × 10 mm); temperature 40°C; pressure 131–139 atm; flow rate 1.50 ml/min. Solutes: 1) corn oil; 2) di(ethylhexyl) phthalate; 3) methoxychlor; 4) perylene; 5) sulphur.

SC-CO_2 relative to conventional organic liquids (Randall, 1984) allows it to replace non-polar liquids such as *n*-hexane in normal phase liquid chromatographic separations. Although there is criticism in the literature regarding the comparison of SC-CO_2 with hexane (O'Neill *et al.*, 1998) the advantage of its incorporation into separation schemes is obvious. This is particularly true where larger quantities of such solvents are used, such as in sample preparation and preparative chromatography.

With respect to the former application, King *et al.* (1994) have shown the effect of substituting SC-CO_2 for the separation of model solute compounds in a modified form of critical fluid size exclusion chromatography. As shown in Figure 9, the separation of model compounds in a test mixture for conventional size exclusion chromatography allows regulation of both solute retention and separation. An equivalent separation to that obtained using methylene chloride as eluent is possible by substituting SC-CO_2 for up to 60 volume percent of the methylene chloride on highly cross-linked micro-particulate styrene-divinylbenzene or pure divinylbenzene "gels". However the separation mechanism is not totally based on solute size considerations, but shows a substantial contribution from solute surface adsorption effects. Based on measurement of the total exclusion and permeation limits of model solutes on the above stationary phases, this mixed retention mechanism is a direct result of substituting SC-CO_2 for methylene chloride for this separation.

These studies were extended by Taylor *et al.* (1996) to develop a new method of sample preparation for the "clean-up" of fat-laden samples for subsequent GC analysis. Sight glass measurements made at high pressures with SC-CO_2 on small columns of size exclusion resins (as described above) revealed that neither styrene-divinylbenzene nor divinylbenzene resins expanded in the presence of SC-CO_2; unlike their expansion in toluene or methylene chloride. As shown in Figure 10, several generic classes of pesticides can be readily separated from a model lipid matrix (corn oil) using the proper ratio of SC-CO_2 to organic solvent. These separations have proven robust and repeatable, particularly when using highly cross-linked, predominantly divinylbenzene resin.

The sorption of SC-CO_2 into polymers is a well-known physicochemical phenomenon and has been characterized by numerous investigators (Shieh *et al.*, 1996a,b; Lambert and Paulaitis, 1991). Springston *et al.* (1986) showed via precise column dead volume measurements that the polysiloxane stationary phases used in capillary SFC swell in the presence of SC-CO_2. Thus, SC-CO_2 dissolution in the stationary phase in SFC plays an active role in solute retention via modification of the stationary phase properties. Sophisticated measurements using isotopically-labelled model solutes by Parcher and co-workers (Strubinger *et al.*, 1991) have confirmed this phenomenon.

The adsorption, as opposed to absorption of SC-CO_2, onto the column stationary phase was studied in the early days of SFC by Sie and Rijnders (1967). Additionally, studies by King *et al.* (1988) show that this phenomenon

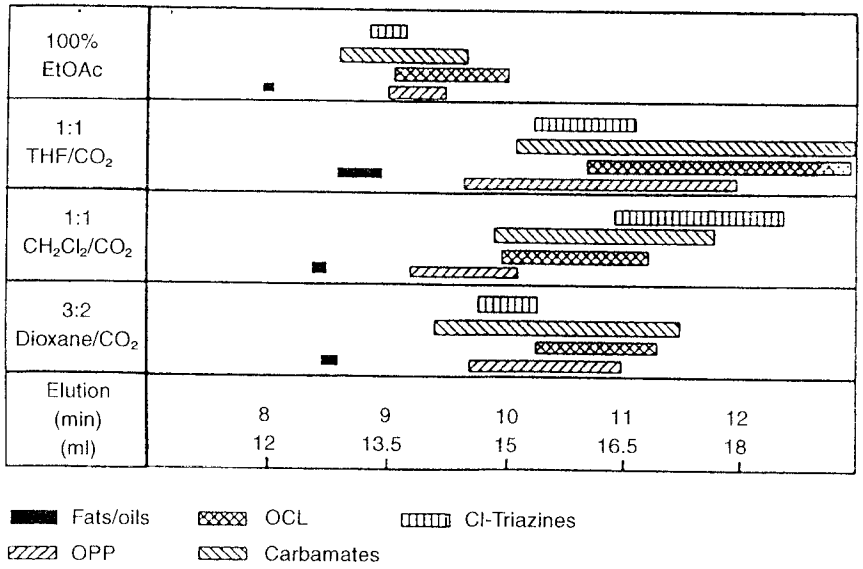

Figure 10. Solute retention volume variation with change in SC-CO_2 – organic solvent composition of the mobile phase.

is influential in eluting solutes and causing solute separation (reversal of relative retention of solutes) for both resinous and solid adsorbent packings. King and others (King, 1987) attribute this effect to the formation of a quasi-liquid layer of CO_2 on the surface of the chromatographic packing; that is, the mobile phase modifies the surface of the chromatographic stationary phase, and becomes an active component of the stationary phase. Again, this observation has been confirmed, most recently by Liu *et al.* (1994).

C. Applications of SFC

Citing examples of applications of SFC is perhaps one of the best ways to document the versatility, advantages and limitations of the technique. In this section we will focus on recent applications in using SFC for analysis, sample preparation, specific niches where coupling SFC with other techniques have proven applicable to lipids, preparative/plant scale SFC and the use of SFC for physicochemical measurements for supercritical fluid technology. The cited analytical applications will embrace both capillary and packed column SFC since about 1995. It should be noted that both types of SFC can yield valuable results in terms of scale-up to the preparative level and physicochemical data. Sample preparation embraces SFC in its low resolution mode, mainly for the separation of lipid classes, the removal of lipid interferences, and in facilitating

certain types of the derivatization by using "reactive" columns that have catalytic properties. Besides coupling SFC on-line with other techniques, tandem arrangements of SFC, particularly with SFE, can help to evaluate the efficacy of a SFE process as well as micro lipid analysis. As noted earlier, the preparative modes of SFC can take several forms, and several of these will be documented along with scale-up to the production plant level. Finally, some instances will be cited of the use of SFC for the determination of physicochemical parameters of importance to SFC and SFE, particularly the determination of binary diffusion coefficients in dense gas media and the surface properties and modification of adsorbents.

1. Advances in analytical SFC

Analytical capillary SFC has found numerous applications for the analysis and characterization of oil and lipid mixtures. These have included raw material characterization, deformulation of commercial products containing oleochemicals, and the analysis of reaction mixtures. Additional areas of application include the detection of product adulteration or deterioration, the fractionation of oligomeric mixtures and, as noted previously, application to very small samples of lipid-containing matter. Overall, analytical SFC is an excellent technique for the initial characterization of natural products, particularly those known to contain lipid moieties.

The SFC research undertaken by Kallio and his collaborators at the University of Turku in Finland has focused on exploiting capillary SFC (cSFC) and SFC coupled with mass spectrometry (MS) for the characterization of lipid mixtures and natural oils of nutraceutical value. For example, γ- and α-linolenic acid (predominant components of evening primrose or blackcurrant seed oils) can be resolved by cSFC using mixed polydimethylsiloxane-bonded columns which are 10 m in length (Manninen *et al.*, 1995a). Separation occurs according to triacylglycerol (TAG) carbon number (ACN), resulting in screening profiles for the various oils having predominantly TAG ACN composition compositions from C_{52}–C_{54}. Similarly, TAGs and fat-soluble vitamins in lipid matrices such as cloudberry oil, have been separated using this technique (Manninen *et al.*, 1995b). These investigators have also combined cSFC with atmospheric pressure ionization-mass spectrometry (API-MS) for separating and identifying the components in blackcurrant oil (Manninen and Laasko, 1997a), milk fat (Laasko and Manninen, 1997) and cloudberry or sea buckthorn oils (Manninen and Laasko, 1997b). Results compared favourably with reported HPLC-APCI-MS data (Byrdwell *et al.*, 1996) for regioisomeric TAGs, which could be optimally separated on SB-cyanopropyl bonded phase capillary columns.

The SFC studies of Hayes have extended the application of cSFC to higher molecular weight TAGs containing oxygenated functional groups as recently

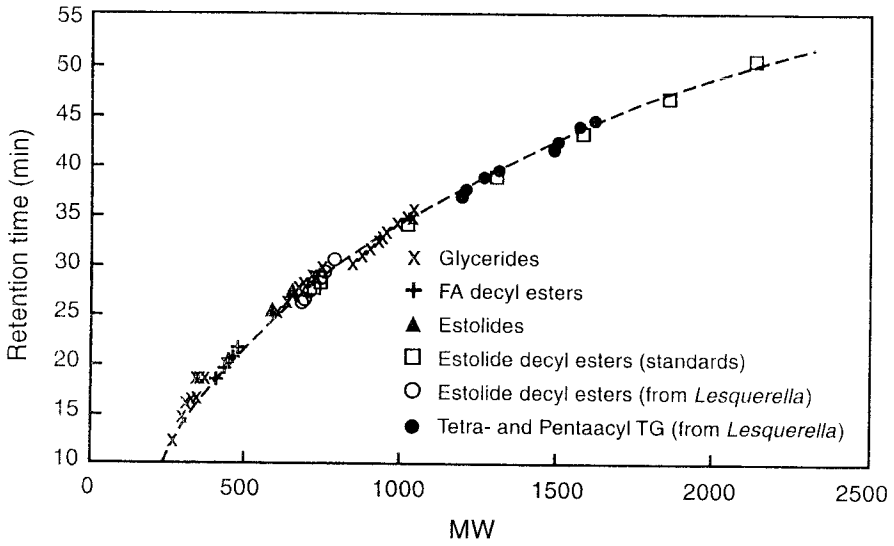

Figure 11. Relationship between retention time of solutes and solute molecular weight as determined by capillary SFC.

reported (Hayes, 1997). Examples of oils containing oxygenated fatty acyl groups that have chromatographed using cSFC include vernonia and lesquerella. Optimal resolution of the TAG moieties is realized at column temperatures greater than 100°C and by using mixed combinations of pressure and temperature program ramps. The resultant retention trends, as depicted in Figure 11, have aided in the identification of complex lipid mixtures containing estolides (Hayes *et al.*, 1995) and polymerized products. For example, the TAG compositions of crambe, meadowfoam, vernonia, and C_{20}-hydroxylated acyl groups in lesquerella oil have been identified from these retention trends (Hayes and Kleiman, 1996b). Reaction mixtures resulting from lipase-initiated esterification of estolides or alcoholic functionalites can also be characterized (Figure 11).

Identification of TAG peaks from free fatty acids (FFA) or estolide peaks in cSFC profiles, as shown in Figure 12, is possible by scrutiny of the shape of chromatographic peak profiles (i.e. TAG peaks are more symmetrical than the FFA and estolide moieties) (Hayes and Kleiman, 1996b), as noted previously (King, 1990). Such results arise from the differing sorption isotherms exhibited by the lipid solutes (analytes) on the chosen stationary phases. It is interesting to note that the above researchers have used cSFC to characterize lesquerella oil lipolysates from a oil extracted off-line by SFE (Hayes *et al.*, 1996c).

A significant body of cSFC and packed-capillary SFC (pcSFC) studies have been undertaken by Borch-Jensen and Mollerup (1996a), particularly for the profiling of marine-derived oils. These investigators note that the suitability of

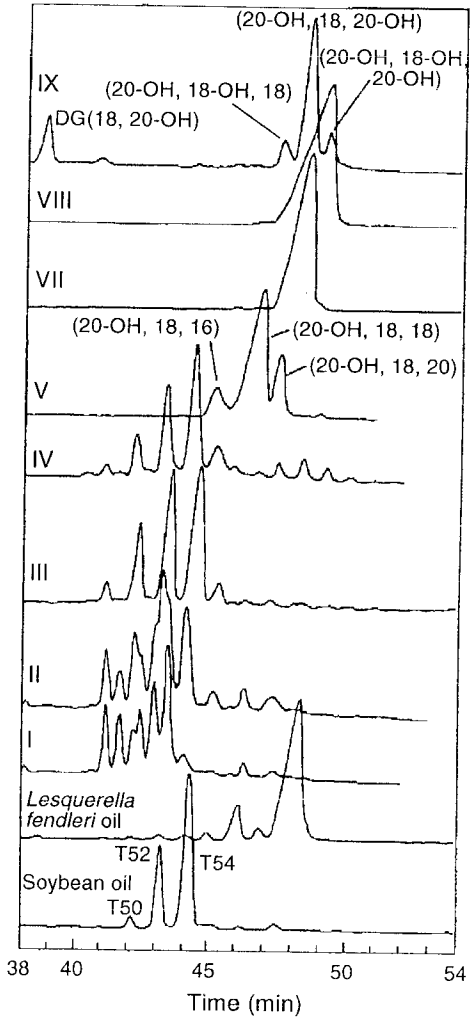

Figure 12. cSFC of lesquerella oil fractions separated on a silica gel column (soybean oil TAGs included for reference; DG = diglyceride).

SFC for oil analysis and characterization depends on ACN and saturated and unsaturated acyl FA composition of the oils. Hence, total resolution of all of the TAG species may not be possible, and result in partially resolved TAG profiles, frequently referred to as "humpagrams". Borch-Jensen and co-workers have also compared the merits of GG/MS analysis utilizing trimethylsilane (TMS) derivatives with cSFC analysis for the determination of hydroxylated acyl fatty acids such as ricinoleic acid in the castor bean. They convincingly argue that

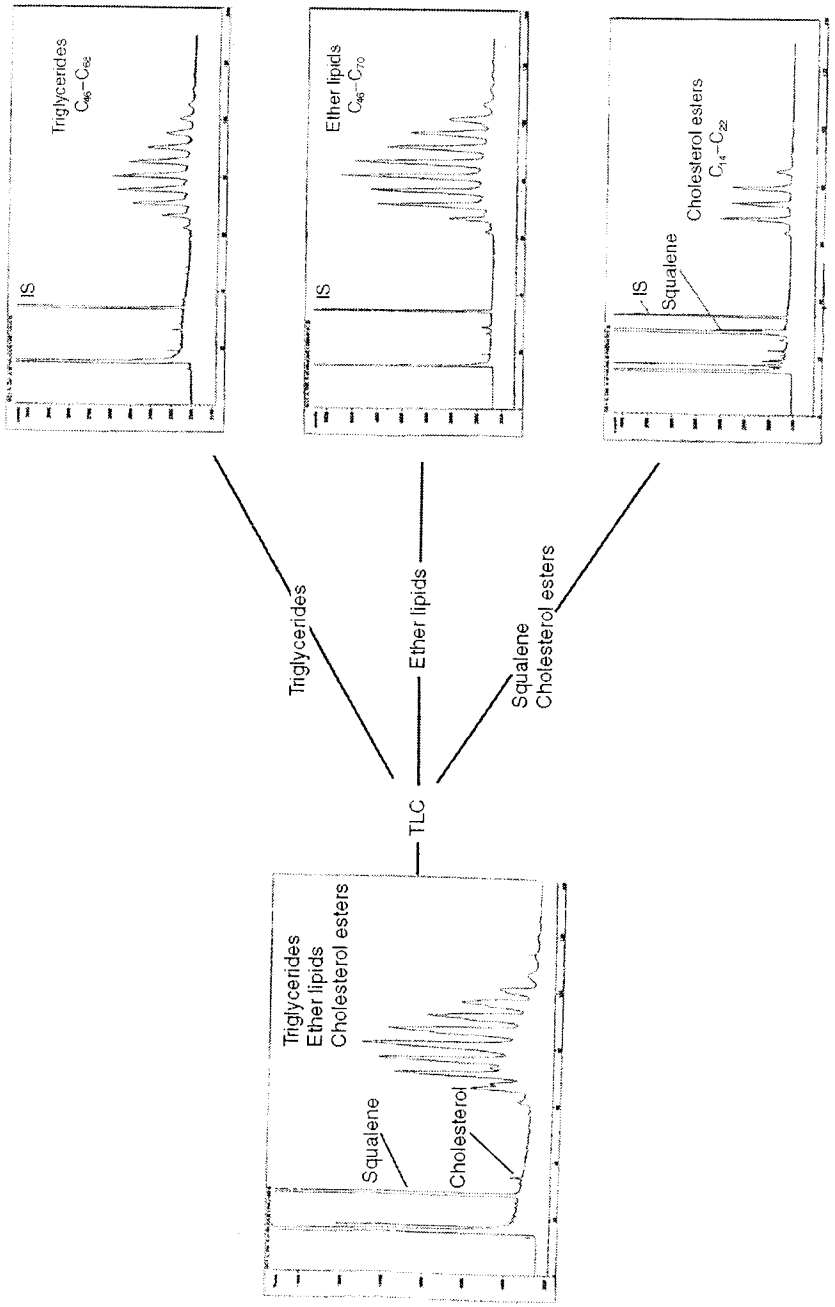

Figure 13. Utilization of thin-layer chromatography (TLC) in conjunction with cSFC for fractionating components in shark liver oil.

SFC assay relative to GC determinations involves less sample preparation, faster analysis and lower temperatures (Borch-Jensen *et al.*, 1997a). They note also that transmethylation of samples followed by GC or GC/MS results in a loss of information with respect to the fatty acid and TAG composition of the chromatographed samples.

As noted in Figure 12, cSFC retention trends permit direct quantitation of minor lipid constituents and non-TAG components such as squalene, cholesterol, cholesterol esters and diacylglycerol ethers. However, a preliminary separation of the lipid components which coelute in the resultant chromatogram may be necessary, as shown in Figure 13. Here, thin-layer chromatography (TLC) provides sufficient fractionation of the interfering lipid classes to permit the use of SFC. Such steps are necessary when analysing shark oils from different species (Borch-Jensen *et al.*, 1997b).

The most informative SFC profiles result from long chromatographic runs (up to 140 min) and the use of conventional GC capillary columns with heavier liquid phase loadings for cSFC, thus permitting more concentrated injections of the oil mixtures to be made for the assay of minor lipid components. An excellent column for this purpose is a 0.2 μ DB-225 liquid phase on a 25 m × 0.1 mm column. This column also works very well for the resolution of the components in a *Euphorbia lagascae*-derived oil (Borch-Jensen and Mollerup,

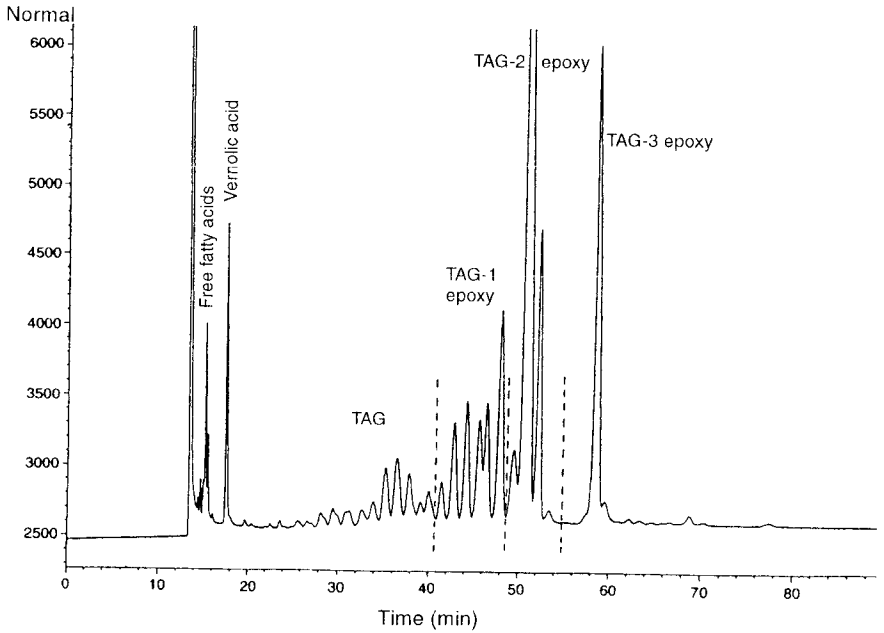

Figure 14. Chromatogram from cSFC analysis of *Euphorbia lagascae* crude oil. TAG: normal triacylglycerols; TAG-*n* epoxy: triacylglycerols with *n* (*n* = 1–3) epoxy fatty acids.

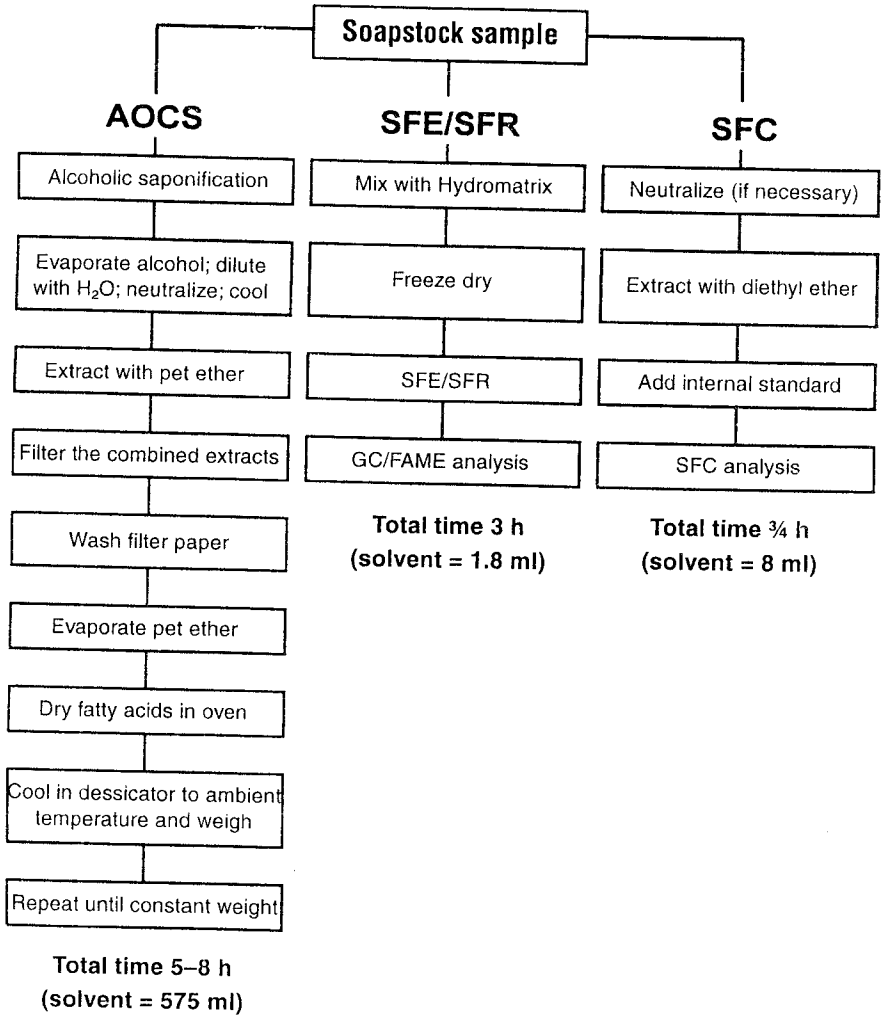

Figure 15. Comparison of the complexity of AOCS official method G3-53 with SFE/SFR method utilizing an enzymatically-catalysed reaction and rapid SFC method.

1996b), which contains vernolic acid and epoxylated-TAGs, as depicted in Figure 14. This column can also be used for FFA derived from *Euphorbia lagascae*. Results for the determination of vernolic acid in both the crude oil (54.9%) and FFA mixture (55.3%) by cSFC compare well with the FAME results (53.5%) from GC assay.

As noted previously, the SFC analysis of FFA can present problems owing to the resulting asymmetrical peak profiles. This problem can occur both in

cSFC and packed column SFC (pSFC), if sufficient attention is not paid to stationary phase support deactivation. To some extent this problem can be avoided by using the FAME derivatives of the FFA, as demonstrated in the studies of Senorans and Ibanez (2002). From the literature, it seems the above problem is better addressed by the use of pSFC, where the choice of packing materials and their treatment with respect to deactivation can be controlled. Nevertheless, FFA analysis using conventional capillary SFC columns can be used to advantage, as demonstrated by the author's research. For example, the FFA content of hydrolysed oils (Holliday *et al.*, 1997) and soapstock (King *et al.*, 1998) have been determined using cSFC. In the latter case, as shown in Figure 15, the SFC-based assay took only 45 min compared to extraction-based assays using either copious amounts of conventional organic solvents or SC-CO_2. The reported SFC assay for FFA is less precise than the more elaborate extraction-based methodology; however, it can quickly ascertain the presence of FFA in soapstock shipments that arrive at plant sites via trailer trucks, and therefore can be used to accept or reject such shipments, thereby avoiding demurrage charges.

cSFC has been demonstrated to be extremely valuable in product deformulation of cosmetic and oleochemical-containing products (King, 1990). As shown in Figures 16a and 16b, key ingredients can be identified in these oleochemical-rich compositions using cSFC and the described mobile phase density programs listed in Table 1. The major lipophilic components in a quencher lipstick formulation (Figure 16a) and a Blistek lip balm (Figure 16b) have been identified by retention matches with standards, and can provide valuable composition information for the formulator of competitive products. It should be noted that the above assays were simply performed by diluting a small amount of the sample in the injection solvent and directly injecting it onto the cSFC column with no attendant sample preparation.

In recent years, packed column SFC (pSFC) has found its own niche in lipid analysis. The excellent research of Lesellier in France demonstrates the potential for applying pSFC in both major component lipid analysis and the analysis of minor/major components in non-saponifiable lipid extracts (Lesellier, 2001).

Table 1. SFC density programs utilized in performing separations

	Program 1 Oven temperature 120°C	**Program 2 Oven temperature 100°C**
Initial density (g/ml)	0.28	0.15
Initial hold time (min)	15	15
Density ramp rate (g/ml/min)	0.006	0.01
Final density (g/ml)	0.66	0.76
Final hold time (min)	15	10
Total run time (min)	90.17	96.72

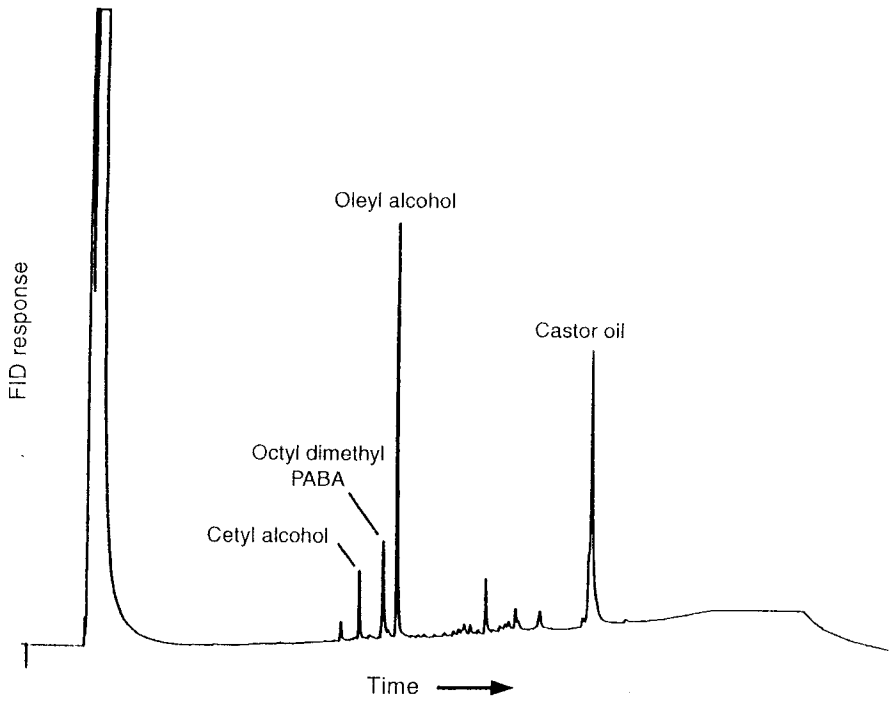

Figure 16a. Capillary SFC separation of lipophilic components in a quencher lipstick formulation.

Ultraviolet (UV) and evaporative light scattering detectors (ELSD) have been the detectors predominantly used in pSFC. Owing to the wide range in detection wavelengths for achieving maximum UV absorbance and molar absorptivities, variable wavelength UV detectors or photodiode array capability are frequently used and desired in pSFC. For example, the following UV wavelengths are used in the detection of these lipid solutes (Lesellier, 2001): free fatty acids (215 nm), tocopherols (290 nm), diglycerides (215 nm), TAGs (215 nm), and 400 nm for carotenoids, since they frequently overlap with the TAG elution patterns in pSFC. It should also be noted that the wavelength for maximum UV absorbance for a given lipid class or compound in SC-CO_2 with or without co-solvents, is not the same as in the liquid organic solvents used in HPLC. Bathochromic shifts frequently occur for various UV functional groups in SC-CO_2 (a function of pressure); hence it is best to determine the wavelength of maximum UV absorbance in the SF mobile phase.

In the 1990s, ELSD found widespread application in HPLC and pSFC for the detection of lipid solutes and other non-chromaphoric analytes. There are excellent reviews on the use of ELSD in SFC by Dreux and Lafosse (1997) and Thompson *et al.* (1998). One of the main problems in coupling an ELSD to SFC

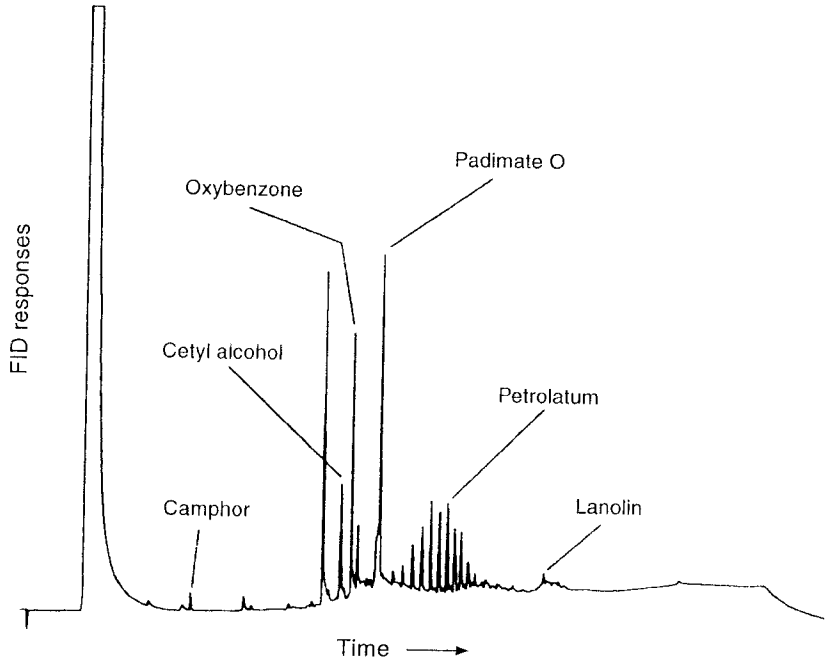

Figure 16b. Capillary SFC separation of lipophilic components in a commercial lip balm.

systems is dealing with the concomitant pressure drop that occurs as the solute-laden mobile phase is decompressed at the end of the column. This can affect the sensitivity of the ELSD and lead to spiking, owing to the formation of CO_2 "snow" particulates entering the detector proper. As shown in Figure 17, there are several alternatives to regulating the pressure on the SFC system prior to the ELSD, including using a capillary silica restrictor, a back pressure regulator, or by introduction of a solvent at the column exit. The latter two methods have the advantage of being independent of the SF flow rate, but can have attendant extra column effects which compromise resolution. The method advocated by Pinkston and co-workers (1991) has much to recommend it when using SFC with ELSD in tandem.

Druex and co-workers have used pSFC-ELSD for the analysis of free bile acids (Villette *et al.*, 1996). pSFC columns were utilized with methanol- or iso-propanol-modified SC-CO_2 eluents, which were further modified with trace quantities of water to improve the symmetry of the chromatographic peaks. The ELSD in this case was modified for low temperature operation to avoid degradation of the analytes. Lesellier has also studied the retention behaviour of TAG solutes on C_{18} packed columns (Lesellier and Tchapla, 1999). Over 15 oils were characterized with respect to their elution profiles, and retention data

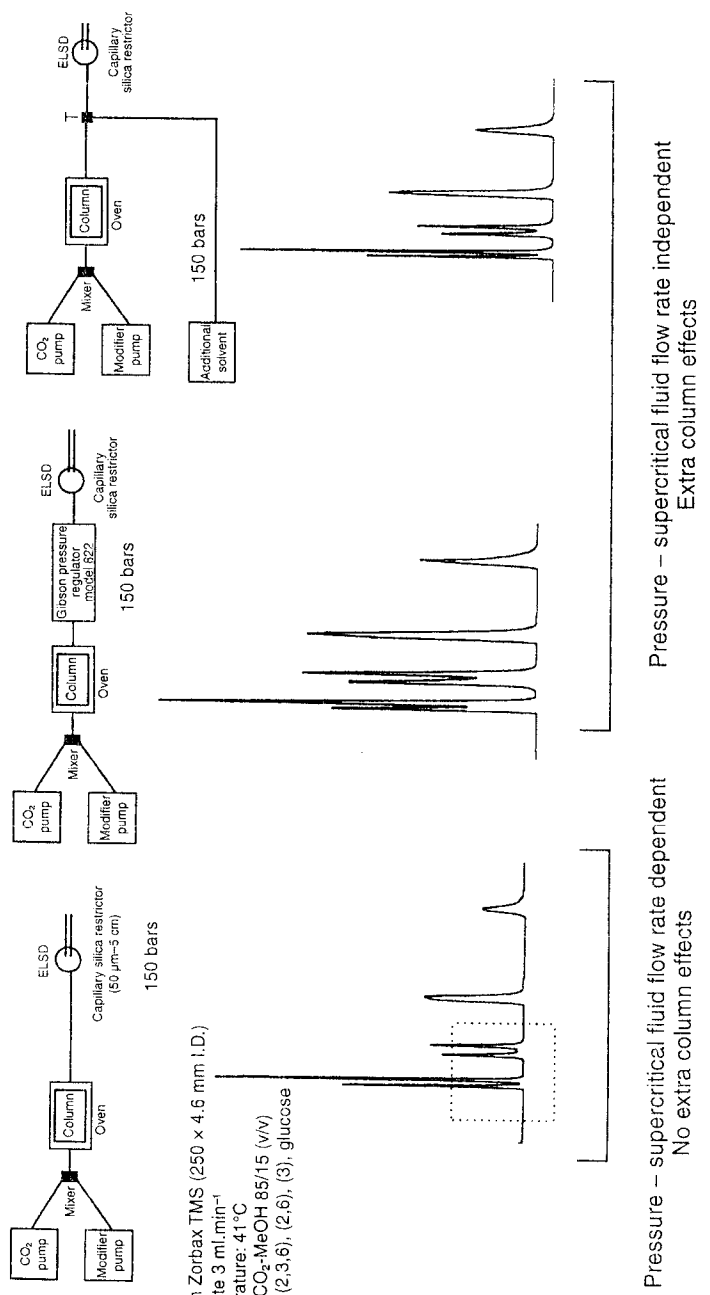

Figure 17. Back pressure regulating options for SFC-ELSD coupled analysis systems.

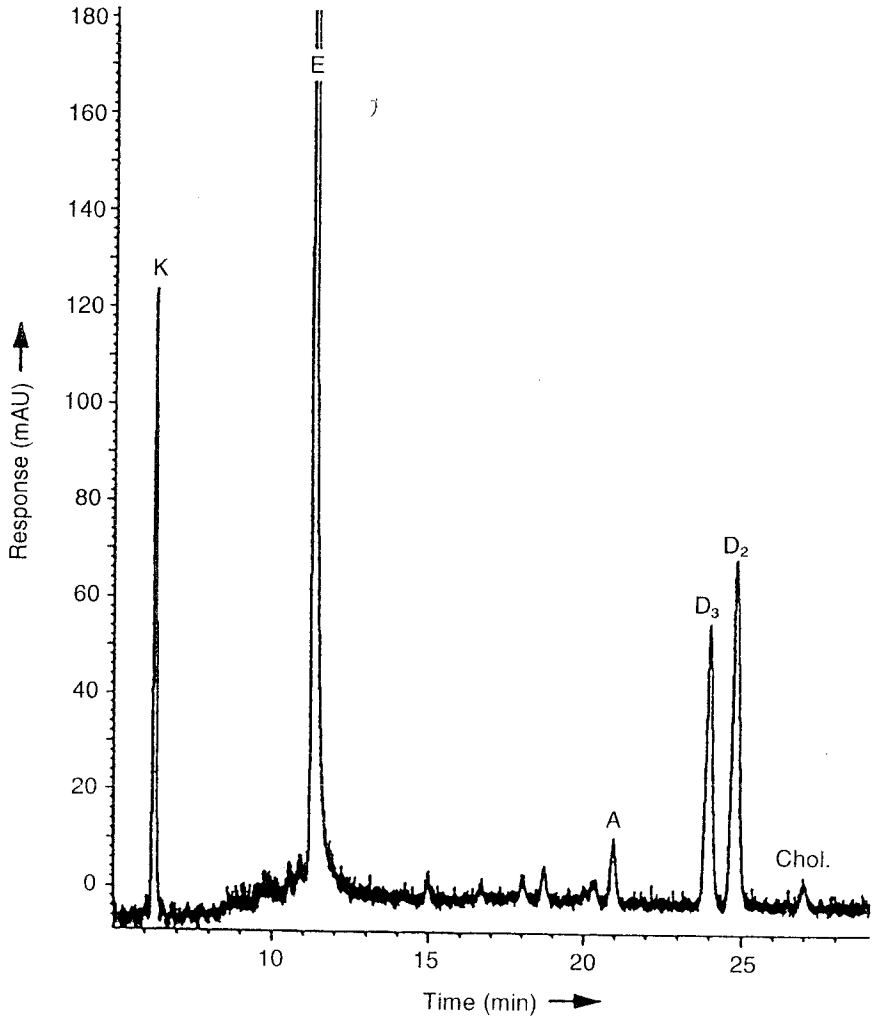

Figure 18. Separation of fat-soluble vitamins and cholesterol by multi-column SFC. Conditions: eluent SC-CO_2/2% MeOH; flow rate 2.5 ml/min; pressure 100 bar; temperature 35°C; column 5 Hypersil, 5 μ (200 × 4.5 mm); detection UV (210 nm).

recorded for 30 individually separated TAGs. The recorded retention trends for solutes is largely a function of their degree of composite unsaturation, similar to trends in normal phase HPLC (npHPLC) and pSFC under sub-ambient conditions.

Snyder and King (unpublished results) have used multiple column pSFC for the separation of fat-soluble vitamins and cholesterol, as shown in Figure 18.

Table 2. Comparison of HPLC and SFC analyses of phospholipids concentrated from soybeans by SFE (normalized %)

Phospholipid	Sample 1		Sample 2		Sample 3	
	HPLC	SFC	HPLC	SFC	HPLC	SFC
Phosphatidylethanolamine (PE)	15.4	14.6	20.8	18.8	14.8	14.6
Phosphatidylcholine (PC)	80.0	82.8	79.2	81.2	80.9	79.2
Phosphatidylinositol (PI)	2.5	3.3	0.0	0.0	3.4	5.4
Phosphatidic acid (PA)	2.1	1.2	0.0	0.0	0.9	0.8

Here a SC-CO_2 eluent modified with 2% methanol at 100 bar and 35°C has been used to separate vitamins K, E, A, D_3, D_2 and cholesterol within 20 min. The separated solutes were detected at 210 nm and separated using 3-Hypersil (200 × 4.5 mm, 5 μ) connected in series. Vitamin A has also been quantified in pharmaceutical preparations by Becerra and co-workers (1999), utilizing SFE for analyte extraction and pSFC for the final determination. A recent comprehensive review summarizes the use of SFC for vitamin separation and assay (Turner *et al.*, 2001).

Snyder, King, and Taylor (unpublished results) also used pSFC for the separation of the major phospholipids (PLs) in soybeans. A Hypersil (200 × 4 mm) column yielded the best separation results at 150 atm, 50°C, using a SC-CO_2 20% ethanol:water (90:10) mobile phase. A quantitative comparison to HPLC on samples extracted from soybeans by SFE is shown in Table 2. The normalized results for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) compare very well with the HPLC results for the same three samples.

There are numerous other examples of the applicability of SFC (in capillary and packed column mode) to lipid analysis, particularly for the assay of minor lipid components. For example, Ibanez *et al.* (1998) separated several carotenoids by sub-ambient pcSFC using a homemade column of silica coated with conventional GC liquid phases such as SE-54 and OV-17. Successful separations were recorded at 300 bar and 10°C using neat SC-CO_2. Similarly, Lesselier *et al.* (1999) studied four column types to optimize the separation between *cis* and *trans* β-carotene. Retention trends for model solutes paralleled those found in normal phase adsorption chromatography on reversed phase sorbents (NARP-HPLC). The developed method could be used to characterize an isomerized β-carotene sample, where all the *trans* isomers, the β-*cis* form, and the 15-*cis*, 9-*cis*-isomers were successfully separated.

Other lipid components, which usually occur in low quantities in natural oils or in lipid concentrates, are amenable to analysis by SFC. Bamba and co-workers (2001) have demonstrated the applicability of pSFC for the separation of polyprenols. They used a CO_2 mobile phase modified with ethanol and a Inertsil ODS-3 column (250 × 4.6 mm, 5 μ) to separate polyprenols from Tochu

(*Eucommia ulmoides*), a woody plant, using detection at 210 nm. These researchers noted that SFC assay of the polyprenol content was more applicable relative to HPLC utilizing UV detection, where a number of interferences are present. Other uses for pSFC for minor constituents in lipid matrices have been investigated by Sorensen and co-workers (1999). They noted that SFC was a facile technique for the analysis of amphilic compounds (such as phospholipids) that affect the quality of rapeseed, soybean, olive and crambe oils. Again, a CO_2 co-solvent (4% acetonitrile) mobile phase at 30 MPa and 40°C using a Spherisorb S3 ODS2 (150 × 4.6 mm, 3 μ) column with detection at 205 nm provided characteristic profiles. Under similar experimental conditions, these same investigators used CO_2 in methanol gradients (4–20%) to separate 12 chlorophylls and their degradation products (Buskov *et al.*, 1999). The above research team has also published elegant studies utilizing SFC for the determination of non-lipid solutes, such as glucosinolates (Buskov *et al.*, 2002a,b), and related ascorbingens in oilseed matrices.

Related UV-sensitive lipid solutes have been separated using SFC. Yarita and colleagues (1994) studied tocopherols on ODS silica-based columns, separating them on an ODS column using a CO_2-methanol mobile phase. The agreement between SFC and HPLC that these researchers obtained for the tocopherol content in four major oilseeds was most impressive (Table 3). Tocopherols were also resolved using pcSFC on a 800 × 0.2 mm column packed with 5 μ C_{18} packing incorporating an 8% modified CO_2 eluent (Senorans *et al.*, 1999). Ampherometric detection was employed to quantify the tocopherols to the 250 picogram level. The resultant analysis was only 20 min long and could be performed without removing the TAGs or unsaponifiables, owing to the specificity of the detector for tocopherols.

SFC can also be used to detect differences in lipid compositions used in food applications. Artz *et al.* (1997) applied cSFC for the characterization and content of heated fats after frying studies. These authors noted that SFC

Table 3. Comparison of SFC and HPLC determination of tocopherols in vegetable oils

Oil	Method	Content of tocopherol (mg/100g)*			
		α-	β-	γ-	δ-
Wheat germ	SFC	259.0	119.0	41.7	3.1
	HPLC	267.0	105.0	35.5	1.6
Soybean	SFC	19.9	3.4	136.0	47.0
	HPLC	19.9	6.4	132.0	46.9
Rice bran	SFC	28.2	1.0	1.2	Trace
	HPLC	27.6	1.4	1.3	Trace
Cottonseed	SFC	133.0	1.2	61.4	Trace
	HPLC	130.0	8.6	58.4	Trace

* Mean value

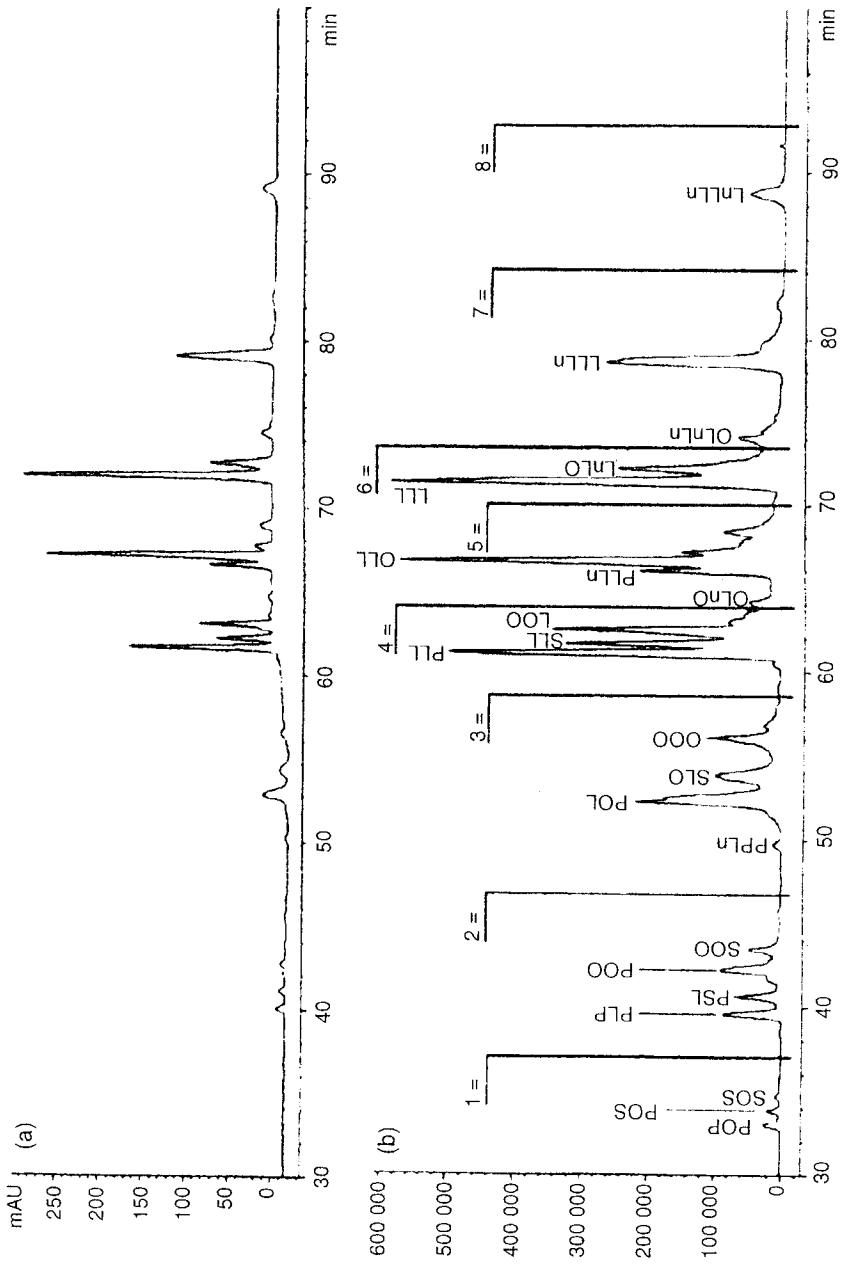

Figure 19. pSFC separation of soybean oil TAGs: (a) UV detection at 210 nm; (b) APCI-MS.

provided a more specific assay for individual TAGs than high performance size exclusion chromatography (HPSEC). Detection of the resultant fatty acids allowed assessment of first-order rate kinetics for the oil decomposition. These researchers also applied cSFC with HPSEC for comparison of low-linoleic, partially hydrogenated soybean oils used to pan-fry hash browns, before and after heating (Soheili *et al.*, 2002). Lee and Hastilow (1999) used cSFC and high temperature gas chromatography (HTGC) to quantify and profile the TAGs in structured lipid compositions intended for food use. Optimal results in this study were obtained on a SB-Methyl (10 m × 100 μ, 0.25 μ i.d.) capillary column, resulting in 45–60 min assay times.

Complex lipid mixtures, particularly those containing a large number of saturated and unsaturated TAGs, have been addressed in both SFC and HPLC using argentation chromatography (Adlof, 1995). Prior to 1995, Blomberg's research team in Sweden (Demirbuker *et al.*, 1992) illustrated how argentation chromatography could be applied in SFC. Using homemade columns containing Nucloesil or other cation exchange packings, over 17 TAG standards as well as the TAG components in corn and cohune oil could be nicely resolved. A summary of this important work is provided in the review by Hayes (1997).

Recently, the argentation principle has been applied by Sandra and coworkers (2002) using pSFC coupled with APCI-MS to characterize the TAGs in vegetable oils. The SFC column was operated incorporating Ag^+ in the mobile phase, similar to the Ag^+ ion-saturated, sulphonic acid-based, strong cation exchange HPLC columns [Nucleosil 100-5A (250 × 4.6 mm, 5 μ)] employed by Christie (1988) to separate TAG isomers in complex lipid compositions. In the Sandra study, the incorporation of Ag^+ mobile phase spray into the mass spectrometer resulted in the enhancement of analyte sensitivity 100 times relative to that experienced utilizing a UV detector ($\lambda = 210$ nm) prior to the APCI-MS. Both $[M\text{-}H]^+$ and $[M\text{-}Ag^+]$ type molecular ions were produced which aided in the identification of the TAGs. This is amply illustrated by contrasting the resulting profiles in Figure 19.

Somewhat unusual applications of SFC in lipid analysis have also been reported. For example, Shen *et al.* (1994) have utilized cSFC to profile such oils as tea, Hanghua, cod-liver, and Jiuxin, which are used in traditional Chinese medicines. Kaneshiro and Clark (1995) applied cSFC (a SB-Biphenyl column) to monitor the action of thermophilic bacteria on the lipid compositions when exposed to the high temperatures and pressures (500 atm) occurring in deep sea vents. Macrocyclic archeo-type core lipids formed under these conditions were separated. Schmeer *et al.* (1996) also incorporated a coupled SFC (using both packed and capillary SFC columns) to characterize the lipid composition of a complex extract of di- and tri-acylglycerols from the tree species, *Commiphora guillaumini*, a native of Madagascar. The interest in this case was to determine the lipid composition that served as a insect attractant. However, it was necessary to couple APCI-MS to the SFC and utilize the CI capability of the

MS to produce $[M+H]^+$ and $[M+NH_4]^+$ to spectroscopically untangle the glyceride components in the SFC elution profile.

2. *SFC in sample preparation*

The use of SFC in the sample preparation mode usually involves the use of much lower resolution chromatography and modes other than elution in its experimental execution using SF fluid media. This implies the use of large particle diameter commodity sorbents similar to those used in lower resolution forms of liquid chromatography or solid phase extraction (SPE) columns. In addition (as will be documented shortly), traditional packed column GC support material has proven valuable in the sample preparation aspects of analytical SFE. Table 4 lists of some of the sorbents that have been utilized for sample preparation purposes. These include diatomaceous earth substrates, silica, aluminas of various chemical types, modified silicas, and synthetic polymeric media (Hawthorne and King, 1999).

In terms of application, sample preparation SFC has been focused on three areas: the fractionation of lipid classes, the removal or retention of lipids as a interfering material in analytical assay procedures, and the use of columnar reactive packings (i.e. catalysts). The author has written several reviews (King, 1998a; Curren and King, 2002) on sample preparation utilizing SFs, to which the reader is referred. Here we shall focus on those techniques and methods which use the principle of SFC to prepare samples for further analysis.

Fractionation of lipid species can be accomplished to a certain extent using analytical SFE and changing the density of the SF. However, because of the similar cohesive energy densities of lipid solutes (King, 2002c), this is not a highly effective method for segregating specific lipids or lipid classes from each other. One exception is the isolation of very polar lipids from non-polar lipids, such as the separation of PLs from TAGs and similar non-polar lipids. PLs have negligible solubility in pure CO_2 (King, 2002c), but can be dissolved in SC-CO_2 by the addition of ethanol as co-solvent (Montanari *et al.*, 1999). Hence, to fractionate lipid species from one another, another separation method (i.e. SFE) must be integrated into the SF-based system. In the case of SFC, this implies the use of sorbents after the SFE step, or their integration into the SFE cell proper. Under either of these scenarios, the sorbents, usually used in a

Table 4. Sorbents used for fractionation of extracts

Aluminas	Silica gel
Silicas	Florisil
Celite	Hydromatrix
Silylated silicas	Synthetic resins

packed columnar format, provide a second mechanism to assist in the desired fractionation of the lipid material.

For example, a two-step method (unpublished results) has been developed to separate and concentrate plant sterols using off-line analytical SFE, with sorbent and analytical SFC for the final analysis. The method was initially developed using a refined, bleached, deodorized (RBD) soybean oil containing known concentrations (0.1–1.0 wt.%) of stigmasterol. Both hexane-extracted soybean oil and soybean oil extracted with SC-CO_2 were used in developing the method. (This technique was also applied to canola, corn and cottonseed oils.) Soybean oil (0.5 g) was mixed with 0.5 g Hydromatrix and added into a 7 ml extraction vessel. Glass wool was then inserted into the cell and 1.5 g NH_2-Mega Bond Elut (Varian, Harbor City, CA) coated sorbent was added. The conditions employed for the initial SFE step were: 5000 psi, 80°C, and a flow rate of 2.0 ml/min for 60 min. The second extraction sequence on the same sample matrix utilized SC-CO_2 with 5% methanol at 4000 psi, 80°C, and a flow rate of 1.0 ml/min for 20 min. Each fraction was analysed by analytical SFC with a SB-Octyl-50 capillary column, isothermally at 100°C, using pressure programming.

Using the above two-step SFE -sorbent fractionation method on a soybean oil containing 0.11% beta-sitosterol, 0.06% stigmasterol and 0.04% campesterol, the initial SFE step removed 95% of the triglycerides. Upon application of the second SFE-sorbent step, the concentration of sterols in the initial extract increased from 0.21% to 25%. Similar results were also achieved on other vegetable oils (Table 5). These results indicate that the two-step fractionation method can produce a substantial enrichment of sterols from seed oils for analytical detection. An extension of this method using four discrete SFE steps and methyl *t*-butyl ether as co-solvent has been reported by Snyder and co-workers (1999).

Li and co-workers (1999) have employed a similar scheme for isolating lipid classes from poultry liver samples, although the main focus of this sample preparation method was to achieve a lipid-free extract for subsequent HPLC analysis. Using extraction conditions between 490–680 bar at 40 and 80°C with CO_2, coupled with an *in-situ* bed of alumina in the extraction cell, they were able to isolate between 18–30% of the total lipid matter on the bed of alumina. HPLC-ELSD analysis showed that the *in-situ* alumina column preferentially isolated the polar lipid species, cholesterol and the free fatty acids, while TAGs and steryl esters were extracted in the SC-CO_2 phase. These researchers noted "that the process of in-line trapping is expected to be dynamic in that retained lipids and target analytes may migrate on the sorbent beds in a adsorption-desorption process as the flow of the SF continues" (Li *et al.*, 1999). Therefore a knowledge of the breakthrough volumes of the individual lipid species and target analytes is important if the SFC-based fractionation is to be optimized.

Table 5. Concentration of sterols in seed oils by supercritical fractionation

Seed oil	Initial amount (%)	Amount after SFE (%)
Corn oil	0.2	21
Canola oil	0.7	33
Cottonseed oil	0.3	28
Soybean oil (hexane)	0.2	18
Soybean oil (SFE)	0.2	25

An example of this separation principle is shown in Figure 20. Retention characteristics of the target analytes (organochlorine pesticides) were determined as a function of the total quantity of SF eluent passed through a sorbent bed of alumina used to retain interfering lipids. As illustrated in Figure 20, the breakthrough of the three organochlorine pesticides from the alumina clean-up sorbent (SC-CO_2 at 250 atm and 50°C) follows a classic sigmoidal frontal breakthrough curve. This elution pattern, expressed in terms of total expanded volume of CO_2 through the sorbent bed, was accomplished using about 0.2 g of sample and 1.8 g of alumina in a 3.5 ml extraction cell (King, 1998b).

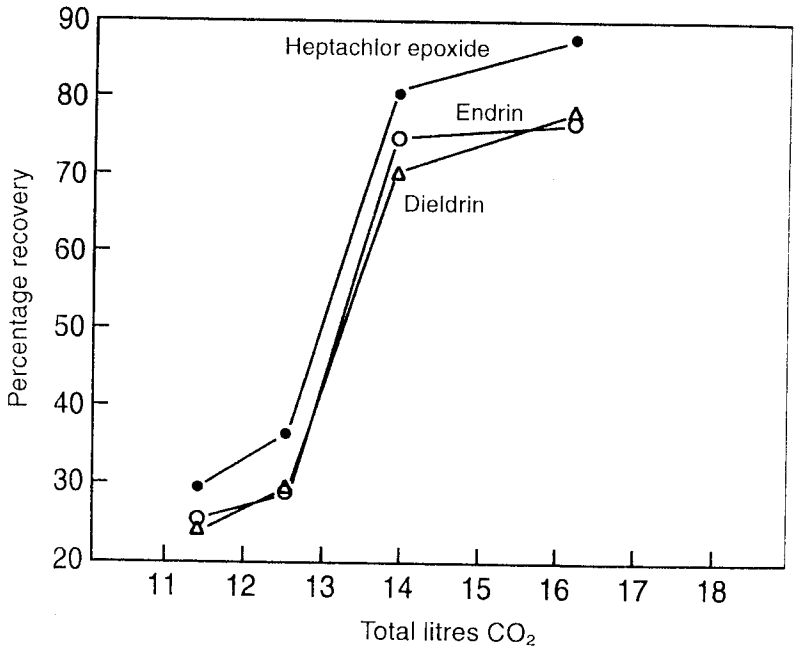

Figure 20. Percentage pesticide recovery versus total expanded CO_2 volume through extraction cell packed with alumina sorbent. Conditions: Lee Model 703 extractor; pressure 250 atm; temperature 50°C; cell volume 3.5 cc.

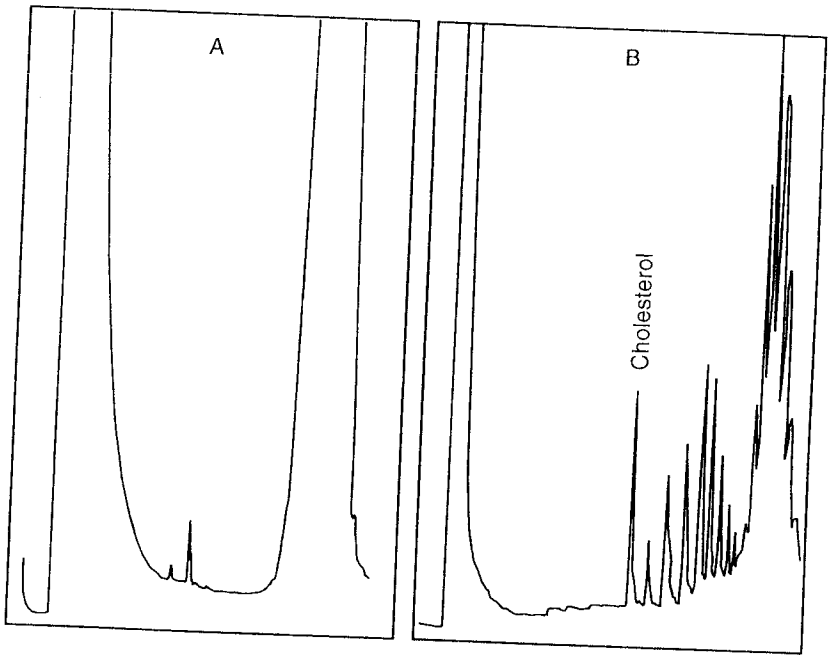

Figure 21. cSFC chromatograms of two SFE fractions consecutively removed from a amino-bonded sorbent in the extraction cell: (A) TAG removal with SC-CO_2; (B) removal of cholesterol and other retained lipid components from sorbent with SC-CO_2-methanol co-solvent. Program conditions for cSFC: 100 atm/5 min; 100–220 atm @ 8 atm/min; 220–236 atm @ 2 atm/min; 236–315 atm @ 10 atm/min; 315 atm/6.1 min; oven temperature 150°C.

Another example of the fractionation of lipids in sample preparation using SFs to achieve a final extract amenable to analysis is depicted in Figure 21. Here an amino-bonded silica (from SPE cartridges) was used to retard sterols selectively relative to other lipid components in the presence of SC-CO_2. Therefore, by using a three-fold excess by weight of this sorbent to sample in the extraction cell, interfering triglycerides could be removed (500 atm and 80°C) from the target analyte, cholesterol. Then, by using 6 vol% of methanol in the SC-CO_2, and the same extraction time and conditions utilized for the neat SC-CO_2 extraction, the cholesterol could be eluted from the sorbent, free of interfering lipids (TAGs), as shown by the cSFC analysis of the collected extract fractions (Figure 21).

One of the other major functions for using SFC in sample preparation is to prepare a lipid-free extract for subsequent analysis. This is particularly important in the analysis of trace analytes in sample matrices containing large amounts of lipid matter (King, 2002a). One approach, as illustrated in Figure 20 above, is to utilize a sorbent that retains the lipid moieties relative to the target analytes. One of the seminal studies of the use of sorbent technology with

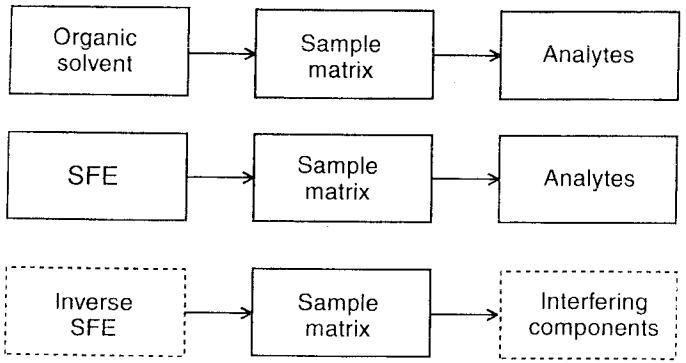

Figure 22. Comparison of inverse SFE with conventional SFE.

analytical SFE for the isolation of pesticides from lipid matter is provided by France *et al.* (1991). Here, neutral alumina, initially thermally-activated, with subsequent adjustment of its final activity level via additional of water, was used to retain interfering lipid moieties while the pesticides (endrin, dieldrin, and heptachlor epoxide) were eluted with high recovery with SC-CO_2 relative to conventional clean-up techniques. The addition of small quantities of co-solvent can aid in the recovery of the analyte using this sample clean-up technique, but care should be taken that breakthrough of the undesired species (lipids) does not occur.

Another option is to use a chromatographic sorbent in conjunction with the SFE step and cell to remove extraneous lipid matter from the sample matrix first. This technique, which retains the target analytes in the SFE cell, was initially developed by the author and his colleagues, and coined "inverse SFE". This concept, relative to conventional SFE and organic solvent-based extraction, is illustrated in Figure 22. Here a sorbent is incorporated into the extraction cell to isolate the target analyte of interest under SFE conditions, while interfering compounds (lipids) are removed by the extraction fluid (SC-CO_2). The analytes are then sequentially eluted by using either neat SC-CO_2 or a co-solvent/SC-CO_2 mixture.

Examples of inverse SFE include: the separation of lipids from leucogentian violet, a coccidiostat found in poultry tissue (King, 1998b); the clean-up of extracts containing aflatoxin M_1 (Taylor *et al.*, 1997); and the reduction of the interfering lipids in extracts containing cholesterol (Figure 20). Maxwell and co-workers (Maxwell and Morrison, 1997; Maxwell *et al.*, 1995, Maxwell and Lightfield, 1998) have made extensive use of this technology to prepare samples in conjunction with analytical SFE for the assay of trace levels of veterinary drugs in biological matrices such as liver and adipose tissue. Both in-line (in the extraction cell) and off-line sorbent traps have been utilized to

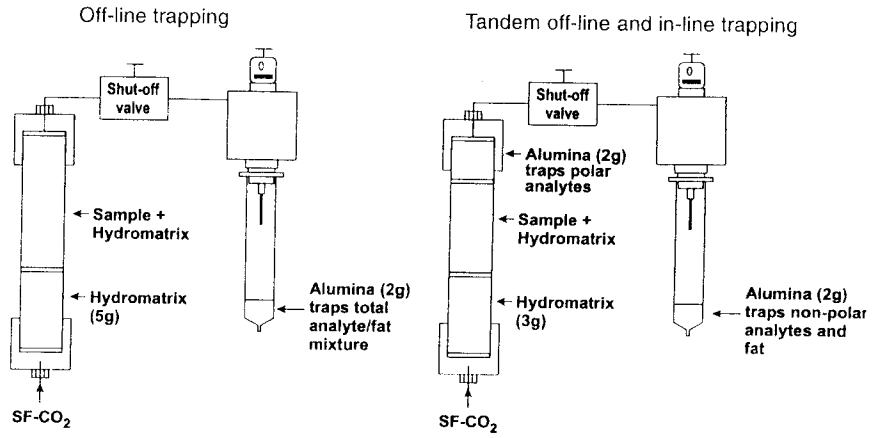

Figure 23. SFE configured for off-line and in-line analyte trapping and fractionation.

isolate the analytes and/or interfering lipids during the extraction/sample clean-up sequence (Figure 23). As illustrated by the right-hand schematic in Figure 23, the *in-situ* alumina column traps either the polar analytes or the polar lipid fraction as mentioned previously. The interfering lipids, or relatively non-polar target analytes that preferentially dissolve in the SF phase, can be collected (after decompression at ambient conditions) on a sorbent-laden cartridge. It is also possible to remove the *in-situ* sorbent column containing the trapped target analytes, and elute them with a solvent for subsequent analysis. Usually the amount of organic solvent required (either as a SF-phase co-solvent, or after the SF step) is minimal. For example, in the analysis of melengesterol acetate (Parks *et al.*, 1996), the traditional sample preparation sequence using required approximately 2.0 l of organic solvent. Using the above SC-CO_2 technique, only 12 ml of methanol was required in the clean-up procedure!

Variants on the above theme have been reported in the literature. The integration of an in-line sorbent to assist in the fractionation of lipids from polar solutes has been reported by Ullsten and Markides (1994), in an on-line SFE system directly coupled to a cSFC for the determination of lipid methyl esters, fatty acids, and TAGs. Ali and Cole (2002) also integrated a one step extraction-sample clean-up sequence, using a C_{18} silica, for selective isolation of non-polar analytes from lipid-containing matrices. In one case they examined, polycyclic aromatic hydrocarbons (PAHs) were determined in commercially-smoked fish, with C_{18} silica retaining 8–15% of the total lipids. Likewise, for the determination of the anti-cancer agents cyclophosphamide and suberoylanide in bovine milk, the silica sorbent retained the above analytes with only 6–18% of the residual lipid content retained on the sorbent. A highly elegant and

automated SFE procedure, incorporating a C_1 sorbent column into an automated supercritical fluid extractor, has been reported by Hopper (1999) for the determination of pesticide residues in fatty tissues. This system permitted the recovery of 86 of 117 organochlorine and organophosphorus pesticides in a multi-residue screening for these toxicants in model vegetable oil matrices (corn, soybean, olive and canola oils) as well as ppb actual incurred pesticide residues in French fries, a fish sandwich, a egg-cheese-ham muffin, and chocolate cake.

It was noted in the introduction to this section that gas chromatographic support materials can be used in SF-based sample preparation methods. In this regard, King and Hopper (1991) used a pelletized diatomaceous earth, Hydromatrix, to prepare a comminuted matrix in a format for extraction using SFE. This material and similar diatomaceous earth-based sorbents, such as Celite 545, are now standard sample preparation materials for analytical SFE. Hydromatrix and its variants permit grinding of the samples (varying in composition from high fat to high moisture content) for introduction as a powdered composite into the SFE cell, and also act as weak or strong adsorbents (depending on the interaction of the target analyte with the sample and diatomaceous earth mixed matrix) towards the components in the sample. Hydromatrix can adsorb approximately twice its weight in water, acting as a mild desiccant, thereby avoiding in certain cases the application of freeze drying to the sample matrix. This unique sorbent parallels the action of other sorbent media used by Barker (2000) in the development of his matrix solid phase dispersion technique, MSPD. Indeed, guidelines for selectively choosing sorbent media for use in SF-sample preparation techniques can be gleaned from the MSPD or SPE literature. Hydromatrix's adsorptive characteristics have been used by the author and his colleagues to isolate lipids or target analytes from various sample matrices, such as trace pesticide analytes from grain matrices, (King *et al.*, 1993b) or, as previously cited, leucogentian violet from poultry fat (King, 1998b).

Chromatographic columns can be employed in the presence of SFs to effect reactions of analytical benefit. These usually involve catalysts supported on porous solids to allow reactions to take place in a flowing stream. Space does not permit an exhaustive discussion of all of the possibilities and studies reported to date. The reader is referred to two detailed reviews on analytical reactions in the presence of SF media (King, 1998a; King and Turner, 2001) for further information. A sampling of the possibilities that supercritical fluid reactions (SFR) offer to the analytical lipid chemist follows.

It was demonstrated several years ago that aluminas used in sample preparation schemes to segregate lipid moieties from target analytes could also be used for methylation of TAGs and fatty acids (King *et al.*, 1992). This is accomplished by using methanol as a co-solvent (reactant) in the SF phase. Excellent results were reported relative to fatty acid methyl ester (FAME) composition of

Table 6. Lipases used in packed columns

Source of lipase	Immobilization method and support
Aspergillus niger	Adsorption – Celite
Geotrichum candidum	Adsorption – Kieselguhr
Rhizopus chinensis	Cell binding – membrane
Rhizopus niveus	Adsorption – Celite
Mucor miehei	Anion exchange resin
Candida antarctica	Adsorption – acrylic resin

oils in the literature, such as evening primrose and soya. The author and others (Snyder *et al.*, 1996) have also reported extensive research on the use of supported lipase catalysts for the transesterification and hydrolysis of target analytes, particularly for the determination of nutritional fat levels in a variety of foods as based on FAME analysis (Eller and King, 1997). A number of supported lipase catalysts are available (Table 6) for the desired transformation of the target lipid analyte. Note that most of immobilized lipases listed in Table 6 are supported on chromatographic adsorbents such as diatomaceous earths or synthetic resin media. An extremely versatile lipase that performs well in the presence of pressurized SC-CO_2 at elevated temperatures is Novozyme 435, derived from *Candida antarctica* (Jackson and King, 1996).

Utilizing this supported lipase and methanol-laden SC-CO_2 between 17–25 MPa and 40–60°C (Jackson and King, 1996), it is possible to convert TAGs to FAMEs readily. Such a reaction has been integrated into a highly automated method by Snyder *et al.* (1996), as shown in Figure 24. Here a bed of Novozyme 435 is placed in the SFE cell after the sample matrix, which is dispersed with Hydromatrix. The SC-CO_2-methanol mixture passes through the sample matrix (solubilizing the lipids) and then over the lipase to form FAMEs. The FAMEs are collected in a mico-trap, then desorbed with a small amount of solvent into a collection vial tray of the automated SFE unit. The cycle is completed by utilizing the robotic arm on the attendant GC to transfer the derivatized samples into the GC column. This unattended, overnight assay, employing integrated SFE-SFR, has been used to analyse the FAME composition of meat, grain and oil samples with an accuracy and precision equivalent to conventional methods (Snyder *et al.*, 1997). It also permits the quantification of both saturated and unsaturated lipids in the above sample matrices as well as the total fat/oil level in the sample matrix.

An extensive study of this lipase-initiated reaction has been reported by Turner and co-workers (2001) for the analysis of fat soluble vitamins in food matrices. Here the hydrolysis of fat soluble vitamins such as vitamin A is accomplished by integrating the supported lipase into the sample-laden extraction cell in the presence of moisture. The lipase-initiated hydrolysis is milder than traditional chemical-initiated hydrolysis at high temperatures, and has

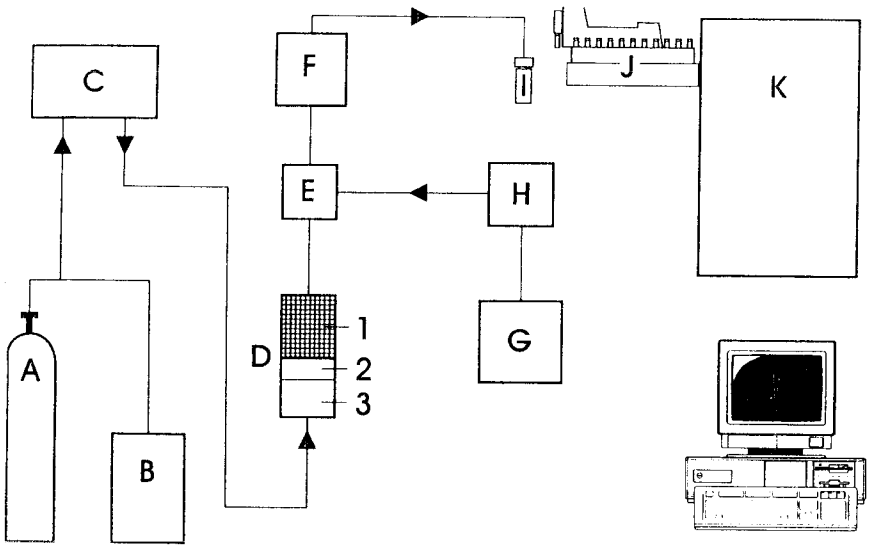

Figure 24. An automated SFE-GC system. Components: (A) CO_2 cylinder; (B) HPLC pump for addition of co-solvent; (C) high pressure pump for CO_2; (D) valve; (E) extraction vessel; (F) analyte trap; (G–H) solvent rinse for analyte trap; (I) collection vial tray; (J) GC robotic arm; (K) GC; (1) sample; (2) glass wool plug; (3) supported lipase.

been applied successfully to milk samples, infant formula and selected meat matrices. Final detection of the hydrolysed moieties is performed by variable wavelength UV and/or fluorescent detection coupled with HPLC. In closing, it should be noted that initial studies on integrating lipase-initiated catalysis with SFE were reported by Berg and co-workers in Norway (Berg *et al.*, 1993, 1997). They integrated a similar supported lipase bed on-line in the extraction cell for the formation of ethyl or butyl esters from TAGs; however, the derivatized solutes were diverted to a SFC or GC for the final analysis.

3. *Preparative and plant scale SFC*

The scale-up of SFC has developed rather erratically, yet it holds considerable promise as a "green", environmentally-benign processing technique. "Preparative" chromatography creates a myriad of different perceptions, experimental execution, and purposes. Preparative SFC is no exception and encompasses a scale-up ranging from milligrams to pounds of material per unit of processing time to, hopefully, tons/unit of time. Among the SF fractionation (SFF) techniques, SFC is perhaps the most capital intensive and requires a higher level of experimental/processing execution by the chemist or engineer. Preparative SFC can range from the very simple concept of mixing an adsorbent

with the sample matrix to enhance the selectivity and resolution of the components to be enriched or separated (in truth, probably not a very practical method amenable for scale-up to the plant level) to ultra-sophisticated pilot plants, using simulated moving bed (SMB) operation.

Since the ultimate aim of many preparative SFC processes is to evolve into a production scale processing plant, it behoves the researcher ultimately to consider the economic consequences inherent in developed SFC processes. The use of expensive columns packings, which might be justified at the semi-preparative scale in a laboratory environment, can create large capitalization costs as the process is further scaled up. For this reason, we have emphasized the use of commodity sorbents as SFC packing whenever possible. Critical to the use of sorbent-based packing materials is their reuse or regeneration after separating lipid components. Any investigation which ignores this factor is susceptible to rejection in scaled-up SFC, where constant replacement of the sorbent from a operating or economic perspective is not feasible. Sorbent regeneration may involve the use of co-solvent with SC-CO_2 or the use of higher densities of SC-CO_2 to remove adsorbed solutes. It should also be noted that one of the critical measures of efficacy in a fractionation process is the amount of target material that can be produced per unit time. Since SF-based fractionation processes are frequently operated at much lower pressure conditions than those utilized in SFE, a trade-off occurs between fractionation efficiency and throughput (the amount of material that can be produced per unit of time).

All the modes of chromatography – frontal, displacement, and elution – have been utilized in the scale-up of SFC. In addition, the application of adsorption technology and the theory that underlies it are seminal to the development of preparative SFC. Consequently, the determination of design parameters (as sorption isotherms and diffusion coefficients), for the implementation of larger scale SFC systems, is extremely important. How such data can be conveniently determined by physicochemical SFC measurements will be documented in the next section. For a discussion of the basic fundamental principles underlying preparative chromatography, the reader is referred to Brunner (1994).

Examples are given below of the different approaches and degree of scale-up that have been realized for preparative/production scale SFC. Much of the past and current focus has been on the enrichment and purification of lipid moieties such as fish oil esters, tocopherols or phospholipids: lipid components having high intrinsic commercial value. Lately, research in this area has been catalysed by developments in the area of nutraceuticals, where there is a demand for enriched concentrates containing lipid components (such as the fatty acid and sterol concentration in saw tooth palmetto berry extracts sold commercially). The selective removal of cholesterol from dairy products, such as butter and milk, can be accomplished by a simple form of SFC. For example, Lim and Rizvi (1996) measured the adsorption breakthrough volume of cholesterol

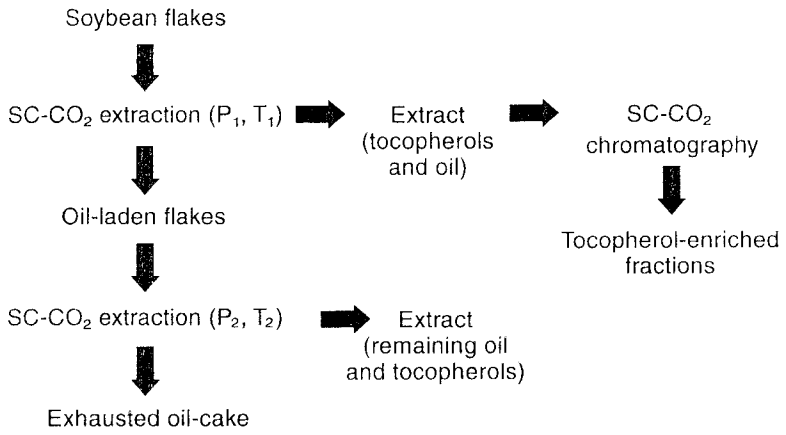

Figure 25. Tocopherol enrichment process using coupled SFE/SFC.

from anhydrous milk fat on magnesium silicate at 40°C and 240 bar. In terms of cholesterol removal (76%) from milk fat, this permitted the processing of 2 g of total fat per gram of adsorbent. The magnesium silicate sorbent, saturated with cholesterol, could be subsequently regenerated under similar conditions to those quoted above using 10% ethanol in SC-CO_2. Mohamed *et al.* (2000) used alumina coupled with supercritical ethane to reduce the cholesterol content of butter oil. The use of ethane increased substantially the amount of butter oil that could be dissolved relative to dissolution in SC-CO_2 at 40, 55, and 70°C, between 8.5–24 MPa. At 40°C and 17 MPa, an extract that contained only 3% cholesterol could be obtained. Experimentally, this was accomplished using a second autoclave downstream from the primary oil dissolution vessel that was packed with alumina.

Tocopherol enrichment from vegetable oils and deodorizer distillates has also been studied, using SFC at various levels of scale-up and technique. Early studies in this area were conducted by Saito and Yamauchi (1990) for the isolation of tocopherols from wheat germ oil. Recycle chromatography was performed using two 250 × 10 mm, 5 μ silica gel columns linked in series. Alpha and beta tocopherols were isolated at 85 and 70% purity levels, although only at the 0.2 g level. The mass balance of the entire SFC process was 102%.

King and co-workers (1996) combined sequentially SFE followed by SFC to enrich tocopherols (Figure 25). The scheme depicted in Figure 25 indicates that a SFE step prior to SFC can be applied for the optimal extraction of tocopherols and additional lipid coextractives (mainly the TAG components) in soybean oil. The initial SFE step concentrates the tocopherols while leaving the predominantly-oil laden flakes available for extraction, perhaps subsequently by a SC-CO_2-based process. The initially-derived extract, enriched

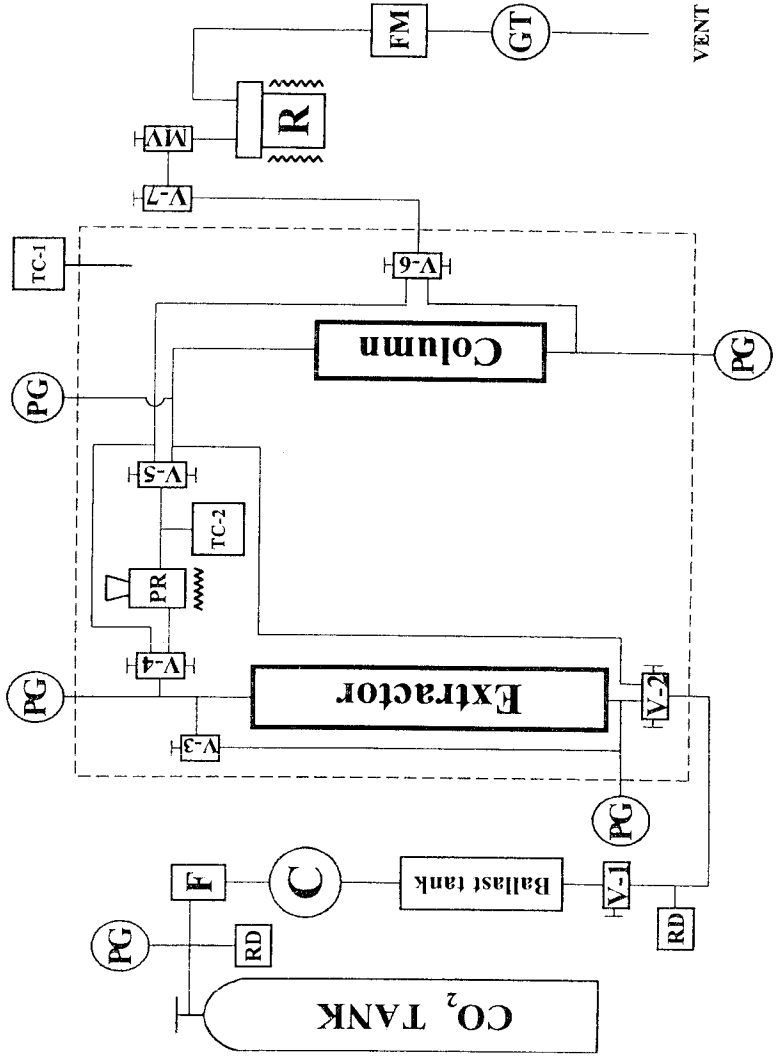

Figure 26. An SFE/SFC processing system.

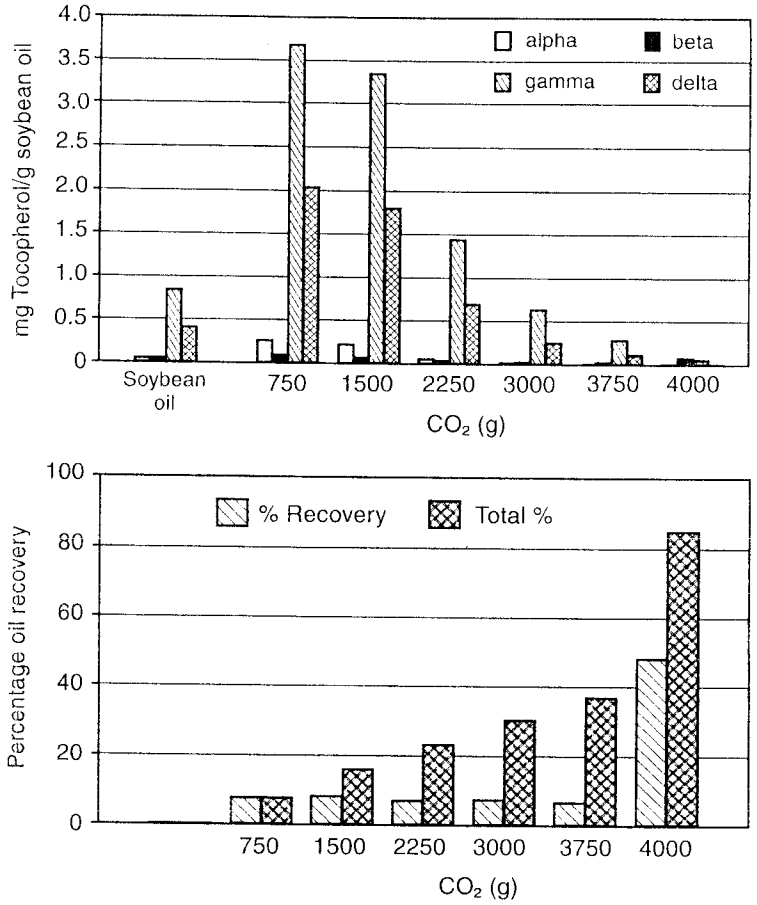

Figure 27. Tocopherol enrichment (a) and oil recovery (b) during the SFE of soybean flakes.

in tocopherol content, is then transported to the top of a chromatographic column, whereby using SC-CO_2 as a eluent in conjunction with a commodity sorbent (silica gel), one can further enrich the tocopherol content of the chromatographed extract by separating it from the background TAG content.

This can be accomplished using the apparatus shown in Figure 26. Here the initial extracts are collected (as a function of time) by passing the SC-CO_2 over the bed of flakes held in the extractor, whereby they are directly deposited at the head of chromatographic column (designated column in Figure 26). Then, by switching the appropriate valves (Vs), the CO_2 flow can be diverted from passing through the extractor to passing through the chromatographic column, where the extracted tocopherols are further separated and concentrated in the

receiver vessel, R. Pressure is maintained on the extractor bed and column by a back pressure regulator (PR) and micrometering valve (MV), respectively.

The initial SFE stage is conducted using a pressure of 25 MPa and a temperature of 80°C. The enrichment of tocopherols occurs up to a reduced mass (g-CO_2/g-soya flakes) of approximately 80. At this point, the oil starts to coextract significantly, diluting the tocopherol-containing extract (King *et al.*, 1996). At this point one would stop the SFE and divert the extract to the chromatographic column for further enrichment. The concentrating effect of this initial SFE stage is illustrated by Figure 27, where the top graph shows the higher levels of tocopherols sequentially collected in the extracts relative to their concentration in the original soybean oil. If one compares the top graph (the enrichment of the tocopherols) with the bottom graph (the coextraction of the soybean oil), one can see that there is a point where continuing SFE is counterproductive.

The SFC stage, using silica gel as the sorbent, also performed at 25 MPa and 40°C, results in further concentration of the tocopherol moieties. If the collection of fractions occurs commensurate with elution of the tocopherols from the column, enrichments of individual tocopherols are possible. Data supporting this are presented in Table 7, where the enrichment factors for the four major tocopherols are shown relative to the tocopherol concentration in the oil from the original soya flakes (oil). The SFE stage produces enrichment factors ranging from 4.33 to 1.83. However, by taking these collected fractions and further enriching them by preparative SFC, enrichment factors (from 30.8–2.4), were obtained, factors which are relatively higher then those obtained by using just SFE.

A similar scheme has been developed for the enrichment of PLs from soya flakes (Montanari *et al.*, 1996; Taylor *et al.*, 2000), except that in this case neat SC-CO_2 is used to extract the soybean oil exhaustively from the flakes matrix. As noted previously, PLs are not solubilized in just SC-CO_2, but ethanol can then be added as a co-solvent to obtain a PL-enriched fraction that can be further by preparative SFC. Table 8 shows the enrichment of PLs obtained by using SFC in sequence with the initial two-step SFE process. Enrichment relative to other eluting constituents (TAGs and other unidentified peaks) ranged from 16.1–2.8 for the SFE stage and 76.8–20.8 after SFC. Two of the

Table 7. Enrichment factors for tocopherols after SFE/SFC*

Tocopherol	SFE stage	SFC stage
α-	4.33	12.1
β-	1.83	2.4
γ-	3.94	15.0
δ-	3.75	30.8

*Relative to starting concentration in soya flakes.

Table 8. Percentage amounts of PLs in extracts derived from SFE and SFC steps*

Phospholipid (PL)	SFE step	SFC step
Phosphatidylethanolamine	16.1	74.9
Phosphatidylinositol	9.2	20.8
Phosphatidic acid	2.8	55.8
Phosphatidylcholine	15.6	76.8

* Relative to other eluting constituents (oil and unidentified peaks).

isolated PLs, phosphatidylcholine and phosphatidylethanolamine, can be obtained at 75% purity levels using this process. Similarly, we developed tandem SFE-SFC processes for enrichment of sterol and steryl esters found in corn bran (Taylor and King, 2002) and fibre (Taylor and King, 2000). It should be noted that these enriched extracts obtained by combining SFE with SFC are intended for the nutraceutical market, where ultra-high purity of the target lipid compound are not necessarily required.

The research group under Professor Brunner at Technische Universität Hamburg–Harburg has also investigated the application of preparative scale SFC for the separation of tocopherol isomers (Depta *et al.*, 1999; Peper *et al.*, 2003). Both elution and simulated moving bed (SMB) chromatography have been applied to separating these isomers, and frontal chromatography using perturbation on the concentration plateaus employed for determining the tocopherol sorption isotherms on Nucleosil and Kromasil silicas. Sorption isotherms of the tocopherols on the silica sorbents were anti-Langmuir in character, as would be expected under overload conditions. Optimized conditions for tocopherol fractionation were found to be 40°C and 20 MPa, utilizing 5% of isopropanol in SC-CO_2. A comparison of the productivity (g tocopherol/hr) for the SMB-SFC process versus the elution mode of separation were 34–56 versus 121, respectively. However, the use of the elution mode of SFC required a higher consumption of the SC-CO_2/co-solvent than the SMB-SFC option.

Perhaps the SFC process that has inspired the most commercial interest and scale-up to a production level capacity is the fractionation and purification of fish oil esters. Studies on both bench scale and pilot plant equipment have been on-going since the early 1990s, and are nicely summarized by Saito and co-workers (1994). Recently Alkio and colleagues (2000) in Finland applied SFC for the purification of polyunsaturated fish oil ethyl esters from tuna oil. A 95% purification of DHA (docosahexaenoic acid ethyl ester) was obtained using neat SC-CO_2 at 145 bar and 65°C and an octadecyl RPHPLC-type of packing. The DHA and EPA (eicosapentaenoic acid ethyl ester) could be simultaneously produced at 90 and 50 wt% purity, respectively. For this separation of DHA and EPA a bonded phase silica is required, yielding a separation factor of 1.40. The production rate (PR) of DHA and EPA per hour based on repetitive injections is given by:

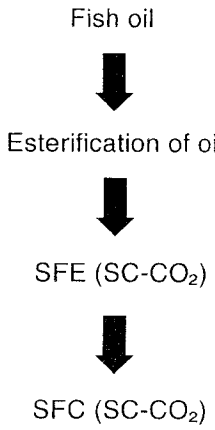

Figure 28. Industrial scale coupled SC-CO_2 SFE/SFC process for fish oil ester production.

$$PR = LR \times C_d \times Y/100\%/t$$

where PR is the specific production rate (g of pure product/kg stationary phase per h); LR is the load ratio (g injected solute/kg stationary phase); C_d is the weight fraction of target solute in the starting material (oil), Y is the yield of the target component (in %), and t is the injection interval in h. The SFC operating cost using the above methodology was estimated to be US$ 550/kg DHA and EPA ester concentrate.

Additional studies by Pettinello and co-workers (2000) have focused on using SFC for the production of EPA-enriched mixtures on both laboratory and pilot plant scale. Using silica gel as the separation medium, Langmuir-type peak behaviour was observed caused by the interaction between the unsaturated fatty acid ethyl esters and the silica gel substrate. In the laboratory scale experiments, 95% EPA ethyl ester purification could be achieved. Extension to the pilot plant level yielded 93% levels of purity. Gravimetric-based yields were 40 and 23% w/w for the laboratory and pilot plant SFC purifications, respectively. As with all SF-based fractionation processes, there was a trade-off between production rate of the target compound and its inherent purity.

Perhaps the most intriguing SFC study and scale-up of EPA and DHA fractionation has been conducted by Lembke in Germany and Spain. Based on Lembke's analytical PhD thesis at the University of Saarlandes (1994), this analytical scale SFC study on the separation of fish oil esters has been scaled-up through a pilot plant installation at KD Pharma in Bexbach, Germany, to a production-scale plant at in Tarragona, Spain. Based on analytical scale separations achieved on 200 × 4 mm amino-bonded silica columns using SC-CO_2 at 170 bar and 37°C, the EPA-DHA SFC process was scaled-up to a pilot

plant level (10–30 l columns), having a fish oil injection pump of 100 l oil/h, and fully automated computer control (Lembke, 1998).

The process is depicted in Figure 28. Chemical esterification on the fish oil is performed initially, then a preliminary SFE step is enacted followed by a proprietary production-scale SFC process. Details of the pilot scale operation are provided by Lembke (1998), and at this scale, the SFC pilot plant can produce 400–500 kg of 95% EPA per year. The author has been privileged to see the actual production plant in Tarragona, Spain, at the Industrias Quimicas Asociadas, SA petrochemical complex, and it is a nice example of developing a PhD thesis into an industrial scale process.

In summary, preparative and production scale SFC in the above described formats may be potentially the most valuable facet of SFC technology. Companies that make bench scale semi-preparative SFC units include Berger Instruments and Thar Designs in the US, while pilot plant and production scale units are produced by NovaSep in France. These companies currently focus on the lucrative pharmaceutical separation market, particularly the separation of chiral compounds. The author believes that one of the most significant applications of production scale SFC as applied to lipids will be centred on the enrichment and purification of PLs, since they are currently purified via large-scale liquid chromatography.

4. *Physicochemical measurements by SFC*

As noted previously, SFC has been utilized for determining physicochemical data that has relevance to processing with critical fluids. Listed below are some, but not all, of the applications of SFC for the determination of specific physicochemical properties:

- diffusion coefficients
- sorption isotherms
- phase distribution constants
- solubility measurements
- critical loci
- partial molar volumes
- virial coefficients

From a historical perspective, the review by van Wasen *et al.* (1980), which emphasises the contributions of Schneider's group in Germany, covers the determination of the physicochemical parameters listed above. A more recent updated review has also been presented by Roth (1991). However, neither of these recommended reviews focus entirely on lipid solutes in SFs.

Knowledge of the occurrence of a phase boundary in systems involving a SF are probably most crucial in terms of assessing the onset of the solubility of a solute in a critical fluid. The measurement of such boundaries or loci depend

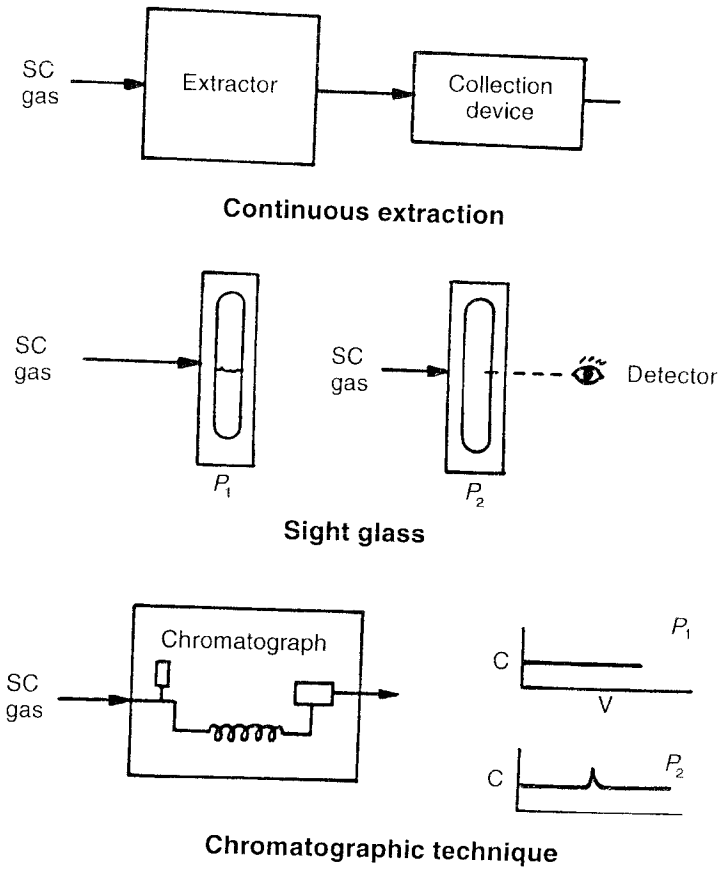

Figure 29. Techniques for measuring the critical loci of mixing.

not only on the composition, but also on the technique employed for their measurement (McHugh and Krukonis, 1994). For example, SFE can be used to determine the pressure dependence of solute solubility in a supercritical fluid (Figure 29). However, if the extract (solute) is collected afterwards for measurement, the pressure associated with the onset of solute solubility in the critical fluid may be subject to error depending on the technique used for measurement. For example, a measurement made with a gravimetric balance (10^{-4} g sensitivity) will not be the same as determining the solute's solubility with the aid of a highly sensitive chromatographic technique (GC, TLC) employing ultra-sensitive detection schemes such as flame ionization detection (10^{-10} g sensitivity). The classic engineering approach to measuring critical loci often employs a sight glass or high pressure optical cell (depicted in Figure 29 for comparison purposes). Without the aid of an optically sensitive

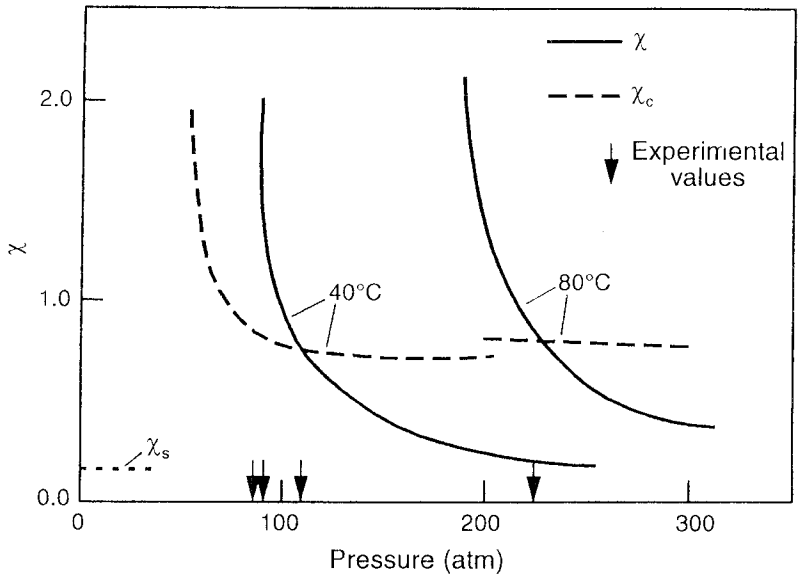

Figure 30. Prediction of miscibility pressure for TAGs in SC-CO_2 from χ vs. pressure plots.

detector to ascertain the onset of critical opalescence, this technique is dependent on the eyesight of the observer and may be subject to slight error. The disappearance of a phase boundary meniscus in going from one pressure to another (P_1 to P_2 in Figure 29) is indicative of solubilization of the solute in the supercritical fluid. Recently, an alternative SFC-based technique has been developed (Ziegler *et al.*, 1995), which depends on the measurement of the appearance of injected solute peaks as they migrate down the chromatographic column or from an injection/extraction chamber inserted into the chromatograph. This can be an extremely sensitive technique as it is inherently more sensitive then the two above-mentioned techniques. However, such a measurement may or may not have relevance to processing applications, since engineers are not always concerned with the isolation or detection of micro or picograms of solute, unless the product being extracted is at low levels in the matrix.

There is also a correspondence between the "elution" pressure and the critical loci for solutes partitioning into the mobile phase of a SFC. For example, the author (King, 1989) has shown that the pressure a solute elutes off a SFC column approximates to the miscibility pressure for these solutes in a SF. If actual SFC experimental elution data are taken and converted to equivalent pressure values from the pressure programming ramp of the SFC unit, then the critical miscibility pressure corresponds to that predicted from a simple application of the Flory–Huggins and Scatchard–Hildebrand solubility parameter theories. Details of the actual computation of the Flory–Huggins interaction χ and χ_c parameters are

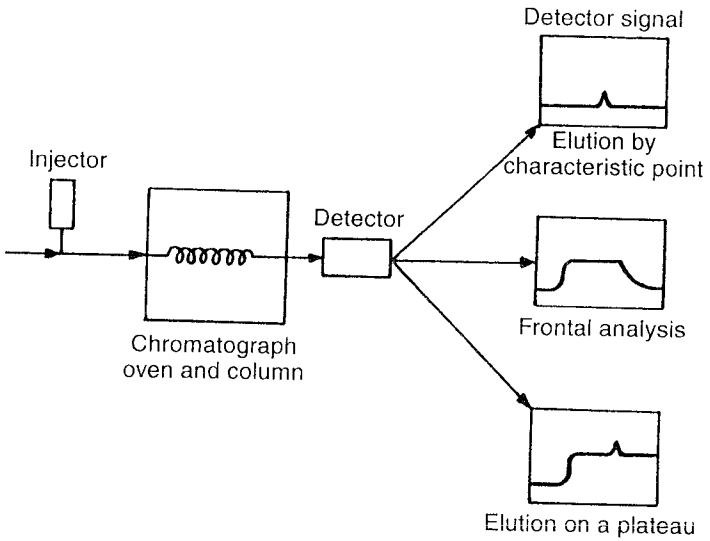

Figure 31. Chromatographic techniques for measuring solute solubilities in SFs.

given in the above publication (King, 1989); however, the graphical interpretation and agreement between the elution pressures and the intercept of the χ_c value of a solute for TAG miscibility in SC-CO_2 is shown in Figure 30. Here the χ and χ_c are in good agreement with the miscibility pressures in SC-CO_2 at 40 and 80°C as determined by gravimetry and SFE. This demonstrates that SFC can be used to determine the onset of lipid solubility in a SF.

The solubility of solutes in SFs can also be measured by chromatographic methods using techniques employed in ambient physicochemical gas chromatographic measurements. These include solubility data at infinite dilution data via the elution by characteristic point method (see Figure 31), or finite measurement of solubilities or sorption isotherms by frontal analysis or elution on a plateau. For example, Yang and Griffiths (1996) used pSFC and cSFC to measure the solubilities and threshold densities of palmitic, stearic, and behenic acids, or cholesterol, in SC-CO_2 as a function of CO_2. Likewise, Kikuchi and co-workers (2002) have used infinite dilute elution SFC measurements to determine the partition ratio, k, partial molar volumes, and binary diffusion coefficients of linoleic and arachidonic acids in SC-CO_2.

SFC retention volume measurements can also prove of value in assessing the interaction between a solute (sorbate) in the mobile fluid phase and a sorbent packed into the column. In this case, the elution by the characteristic point method should give a symmetrical Gaussian peak shape at zero surface coverage on the adsorbent, or asymmetric profiles at finite surface coverages. As noted in Figure 32, the peak maximum of the ideal Gaussian peak profile

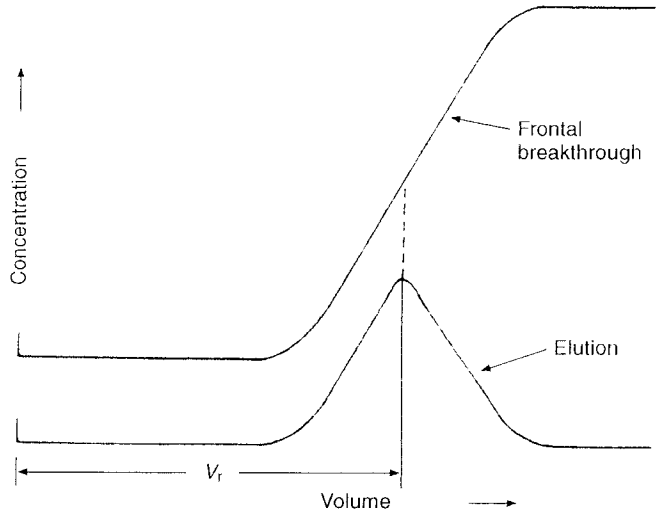

Figure 32. Relationship between the peak maximum of an elution profile and the mid-point of a frontal breakthrough curve.

corresponds to the 50% breakthrough volume of a finite concentration profile continuously fed into the column sorbent bed (i.e. frontal analysis). Therefore the peak maximum retention volume, and its variation with fluid pressure and temperature, can be used to approximate the breakthrough volumes of sorbates being transported down a sorbent-filled column in the presence of a critical fluid mobile phase.

Differences in the breakthrough volumes (BTVs) of various compounds can serve as the basis of their separation via SFC or, more importantly, their retention or lack thereof by the sorbent media. Figure 33 shows the breakthrough volumes of two pesticides, ethion and DDT, for elution from alumina at 60°C and at various pressures. Clearly DDT is retained longer than ethion, although ethion's retention volume (time) would limit the use of this particular sorbent for trapping these two pesticides. As noted previously, breakthrough volumes of target analytes relative to potential coextracted lipid material is

Table 9. Breakthrough volumes (BTV) for different sorbates on XAD-7 sorbent

Measurement	Sorbate-2,4 decadienal	Sorbate-2-pentylfuran
Specific BTV of sorbate from SFC (ml CO_2/g-sorbate)	22.9	6.27
BTV on large XAD-7 column (l)	14.9	4.08
Breakthrough time on XAD-7 (min)	33.6	9.41

Conditions: SC-CO_2 flow rate = 0.443 l/min, 170 atm, 80°C, resin weight = 650 g.

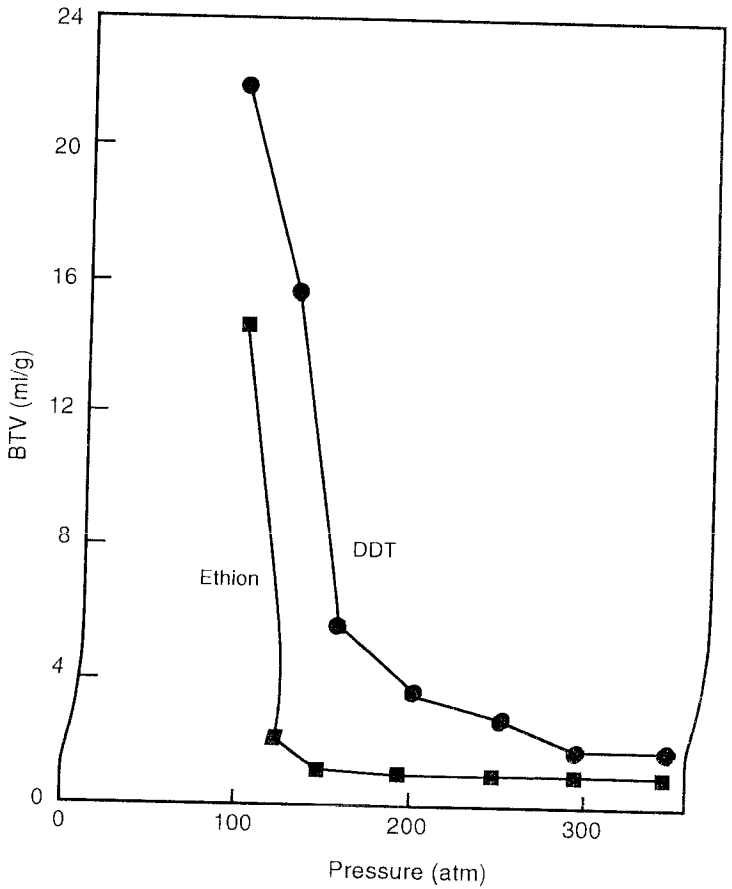

Figure 33. Breakthrough volumes (BTVs) for chlorinated pesticides on alumina as a function of pressure. Conditions: temperature 60°C.

critical to optimizing a sample preparation method involving the use of sorbents.

Breakthrough volumes are also important in process design considerations. Table 9 lists breakthrough volume (BTV) characteristics of relative volatile compounds which are decomposition products from lipid oxidation on a cross-linked polystyrene/divinylbenzene resin, XAD-7 (King *et al.*, 1988). These compounds (adsorbates) are contained in a partial decompressed SC-CO_2 stream after the SC-CO_2 has undergone continuous recycle over a seed oil bed contained in a SFE unit. The adsorbent resin, XAD-7, was being used to remove these odoriferous compounds from the recycle circuit for the SC-CO_2 in order to avoid contaminating the extracted meal. Here the BTVs for the adsorbates, 2, 4 decadienal and 2-pentylfurans as determined by SFC, were

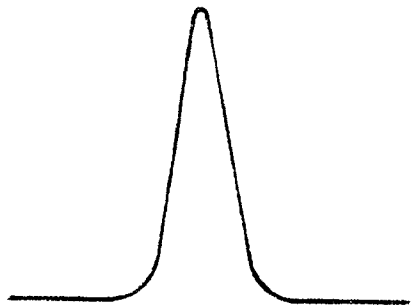

Figure 34. Relationship between solute binary diffusion coefficient and peak shape in SFC.

Table 10. SFC measurement of lipid-fluid D_{12} in SC-CO_2

Lipid solutes	Pressure (MPa)	Temperature (°C)	Reference
C_{18} fatty acids Mixed glycerides Methyl and ethyl oleate	25–36	40–60	Rezaei and Temelli (2000)
$C_{18:0}$, $C_{22:0}$, $C_{22:0}$ ethyl esters $C_{20:5}$, $C_{22:6}$ methyl esters	9.5–21	35–45	Long *et al.* (1991)
C_{16}–C_{24} unsaturated methyl esters	14–34	35–55	Funazukuri *et al.* (1991)
Vitamin K_3 Ubiquinone (UQ-10) α-Tocopherol β-Carotene	9–30	40	Funazukuri *et al.* (2002)

then used to estimate the lifetime of the XAD-7 adsorbent bed in the SFE process. By having knowledge of the specific retention volume (hence the BTV per g of adsorbent at operating conditions), one can estimate the breakthrough volume for any flow rate of the SF through the sorbent bed, and hence the service lifetime before the sorbent needs replacement or regeneration.

The measurement of diffusion coefficients by chromatographic band broadening techniques using SFs at elevated pressure has been practised for over four decades. A recent publication by Rezaei and Temelli (2000) is recommended to those interested in the technique. SFC measurement of binary solute-SF diffusion coefficients (D_{12}) in simplest terms involves using a tube having a specific geometric criterion (e.g. column radius, r), in which the peak dispersion

of a pulse injection of a solute into an open chromatographic column is related to its diffusion during transience in the empty column by the equations noted in Figure 34. Here σ, a measure of the standard dispersion of the resultant chromatographic profile, can be divided into the length of the column (l) to obtain a theoretical plate height, H. The velocity of the carrier fluid, v, is then experimentally varied to obtain the dependence of H on v, from which the binary diffusion coefficient of the solute in the critical carrier fluid can be computed. A good database exists for various solutes at infinite dilution in several SFs (He and Yu, 1997), but data at finite concentrations is lacking. Table 10 provides several examples of the use of SFC to measure D_{12} values of lipid solutes in SC-CO_2 under specific experimental conditions.

D. Conclusions and resources

1. The future of SFC

In this review, we have attempted show the versatility and wide applicability of SFC for the separation and characterization of lipid materials. This has included non-analytical applications such as its use as a low resolution separation technique in sample preparation and for the determination of physicochemical data of importance to SF technology. When SFC is used for the analysis and characterization of lipids, it provides additional data to that obtained from GC or HPLC alone. Analytical SFC is particularly applicable to the analysis of higher molecular weight lipid moieties, such as mixed glyceride compositions ranging from 200–900 in molecular weight. Both cSFC and pSFC lend themselves to lipid assays, particularly when combined with the FID and ELSD. Scale-up of analytical SFC separations is more easily accomplished from the pSFC mode, although both types of SFC can be used for physicochemical property determination as illustrated in the previous section.

The use of low resolution SFC in preparative separations and sample preparation should receive more emphasis since, by using environmentally-benign SC-CO_2, it avoids the use of organic solvents and their attendant expense and laboratory personnel exposure. In this regard, most normal phase LC or HPLC methodology can be converted to a SFC-based method using neat SC-CO_2 or CO_2 with a minimal amount of co-solvent. The availability of analytical SFC instrumentation can be somewhat variable, although additional applications such as the separation of chiral compounds, oligomeric surfactant mixture, and group class fractionation in the petroleum industry assure a market for these instruments. Hopefully, this review has provided some additional examples for using SFC on both analytical and process scales for the reader to consider.

Abbreviations

APCI	atmospheric pressure chemical ionization
BTV	breakthrough volume
cSFC	capillary supercritical fluid chromatography
ELSD	evaporative light scattering detection
FAME	fatty acid methyl ester
FFA	free fatty acids
FID	flame ionization detector
FTIR	Fourier Transform infrared
GC	gas chromatography
HPLC	high performance liquid chromatography
HPSEC	high performance size exclusion chromatography
HTGC	high temperature gas chromatography
LC	liquid chromatography
MS	mass spectrometry
npHPLC	normal phase high performance liquid chromatography
PA	phosphatidic acid
PAH	polycyclic aromatic hydrocarbon
PC	phosphatidylcholine
pcSFC	packed-capillary supercritical fluid chromatography
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PL	phospholipid
pSFC	packed column supercritical fluid chromatography
RBD	refined, bleached, deodorized
SC-CO_2	supercritical carbon dioxide
SF	supercritical fluid
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SFF	SF fractionation
SFR	supercritical fluid reactions
SMB	simulated moving bed
SPE	solid phase extraction
TAG	triacylglycerol
TAG ACN	triacylglycerol carbon number
TLC	thin layer chromatography
TMS	trimethylsilane
UV	ultraviolet

Books related to SF technology

For the reader interested in learning more about the properties and uses of SFs, the following texts and reviews are recommended for further reading.

Analysis with Supercritical Fluids: Extraction and Chromatography, B. Wenclawiak (ed), Springer-Verlag, Berlin, Germany (1992).
Analytical Supercritical Fluid Chromatography and Extraction, M.L. Lee and K.E. Markides (eds), Chromatography Conferences, Inc., Provo, UT (1990).
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Websites on SFC and the use of SFs

The following are interesting websites that the reader is invited to peruse for more information about SF technology.

www.supercritical.co.nz	(SCENZ/Wells Investments, NZ)
www.aphios.com	(Aphios Corporation)
www.kist.re.kr	(Korean Instit. Sci. Tech.)
www.criticalprocesses.com	(Express Limited, UK)
www.aromtech.com	(Aromtech, Finland)
www.simsxt.com	(Marc Sims Inc.)
www.yakimachief.com	(Yamika Chief, Hops; contract SFE)
www.appliedseparations.com	(Applied Separations Inc.)
www.separex.com	(Separex Corporation, France)
www.lavipharm.com	(Fine Particle Technology, NJ)
www.usnutraceuticals.com	(U.S. Nutraceuticals, SF processor)
www.noractech.com	(Norac, Canada)
www.itc-cpv.fzk.de	(Forschungszentrum Karlsruhe)
www.supramics.com	(Supramics, HP CO_2 concrete)
www.ecoplanning.fi	(Chematur, Sweden)
www.natex.sfe.co.at	(Natex, Austria)
www.ErwinSchuetz@t-online.de	(Consultant, Germany)
www.flavex.com	(Flavex, Germany, SF processor)
www.uhde-uht.com	(UHDE, Germany, plants)
www.bpd.co.uk	(Bradford Particle Design, England)
www.thardesigns.com	(Thar Designs, equipment, plants)
www.ourworld.compuserve.com/NATECO_2	(NATECO2, SF processor)
www.wista.de	(Contract SF and HP research)
www.nottingham.ac.uk/supercritical/	(Academic laboratory)
www.pdcmachines.com	(Equipment, pilot plants)
www.phasex4scf.com	(Phasex, contract R and D)
www.arkopharma.com	(Arkopharma, France, SF processor)
www.wenger.com	(Wenger Inc., extruders)
www.tu-harburg.de	(Academic laboratory)
www.flsmiljo.com	(FLS Miljo, Denmark, SF wood)
www.pmiapp.com	(Advanced Pressure Products)
www.isco.com	(Isco, Inc., Instrumentation)
www.leco.com	(Leco Corp., Instrumentation)
www.scfluids.com	(Supercritical Systems, cleaning)
www.praxair.com	(Praxair, contract R and D)
www.micell.com	(Micell, CO_2 dry cleaning)
www.hangersdrycleaners.com	(Hangers Cleaners)
www.hitex@supercritical.com	(HITEX, SF processor)
www.phosphotech.com	(SF, derived phospholipids)
www.novasep.com	(Production scale SFC)
www.intercal.com	(SF, derived products)
www.bergersfc.com	(SFC instrumentation)
www.gilson.com	(SFC instrumentation)
www.scrub.lanl.go	(Los Alamos Superscrub)

Type in "supercritical fluid" at http://search.excite.com/info.xcite/ and watch all of the websites come rolling out!

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